UNIVERSIDADE DE LISBOA
FACULDADE DE MOTRICIDADE HUMANA

THE EFFECTS OF LEUCINE METABOLITES ON PERFORMANCE, BODY COMPOSITION AND BIOCHEMICAL MARKERS OF MUSCLE DAMAGE AND INFLAMMATION

FILIPE JOSÉ NOIA TEIXEIRA

Orientador: Prof.ª Doutora Cristina Paula Fidalgo de Negreiros Monteiro Bento

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Tese especialmente elaborada para obtenção do grau de Doutor em Motricidade Humana na especialidade de Fisiologia do Exercício

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Júri:

Presidente: Doutor Francisco José Bessone Ferreira Alves, Professor Catedrático e Presidente do Conselho Científico da Faculdade de Motricidade Humana da Universidade de Lisboa

Vogais:

-Doutor Stuart Martin Phillips, Canada Research Chair (Tier 1), McMaster University, McMaster Department of Kinesiology, Hamilton, Ontario, Canada

-Doutora Analiza Mónica Lopes Almeida Silva, Professora Auxiliar com Agregação da Faculdade de Motricidade Humana da Universidade de Lisboa

-Doutor Vítor Hugo da Costa Gomes Moreira Teixeira, Professor Auxiliar da Faculdade de Ciências da Nutrição e Alimentação da Universidade do Porto

-Doutor Paulo Alexandre Silva Armada da Silva, Professor Auxiliar da Faculdade de Motricidade Humana da Universidade de Lisboa

-Doutora Cristina Paula Fidalgo Negreiros Monteiro Bento, Professora Auxiliar da Faculdade de Motricidade Humana da Universidade de Lisboa

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MUITO OBRIGADO.

Table of Contents

Tables and figures

Tables List

Figures list

Abbreviations

µL	Microliter
1RM	1 repetition maximum
3-MH	3-methylhistidine
4E-BP1	4E binding protein 1
ACTH	Adrenocorticotrophic hormone
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AMPK	AMP-activated protein kinase
ASCT2	Alanine-serine-cysteine-preferring transporter 2
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
B	Baseline
BCAAs	Branched-chain amino acids
BCAT	Branched-chain amino acid aminotransferases
BCKDC	Branched-chain α-keto acid dehydrogenase enzyme complex
BIA	Bioelectrical impedance analysis
BIS	Bioelectrical impedance spectroscopy
BM	Body mass
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
$CD4^+$	Cluster of differentiation 4
CK	Creatine kinase
cm^2	Centimeter square

CONSORT	Consolidated standards of reporting trials
COX-2	Cyclooxygenase 2
CRP	C-reactive protein
CSA	Cross-sectional area
CV	Coefficient of variation
dL	Deciliter
DLD	Daily living disability
DM	Diabetes mellitus
DOMS	Delayed onset muscle soreness
DXA	Dual-energy X-ray absorptiometry
EAAs	Essential amino acids
ECoAH	Enol-CoA hydrase
ECW	Extracellular water
EDTA	Ethylenediaminetetraacetic acid
eEF2K	Eukarotic elongation factor-2 kinase
eIF2α	Eukaryotic initiation factor 2 α
EIMD	Exercise-induced muscle damage
FFM	Fat free mass
fL	Femtoliter
FM	Fat mass
FSR	Muscle protein fractional synthetic rate
GDH	Glutamate dehydrogenase
GH	Growth hormone
HDL	High-density lipoprotein
HMB	β-hydroxy-β-methylbutyrate
HMB-Ca	β-hydroxy-β-methylbutyrate calcium

HMB-FA	β-hydroxy-β-methylbutyrate free acid
HMG-CoA	β-Hydroxy-β-methylglutaryl-CoA
HsCRP	High-sensitivity C-reactive protein
HSP	Heat shock proteins
ICW	Intracellular water
IFN-γ	Interferon gamma
IGF-1	Insulin-like growth factor 1
IL-1	Interleukin 1
IL-10	Interleukin 10
IL-1β	Interleukin 1 beta
IL-6	Interleukin 6
IU	International unit
IVA-CoA	Isovaleryl CoA
kcal	kilocalorie
kg	Kilogram
kHz	Kilohertz
KICD	α-KIC dioxygenase
LAT	L-type amino acid transporter
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
LST	Lean soft tissue
MC-CoA	β-methyl-crotonyl-CoA
mg	Miligram
mL	Milliliter
mmol	Millimole

MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
MT	Muscle thickness
mTORC	Mammalian target of rapamycin complex
mTORC1	Mammalian target of rapamycin complex 1
MVC	Maximal voluntary contraction
MyoD	Myogenic differentiation factor
N	Newton
NAD^+	Nicotinamide adenine dinucleotide
NF-κB	Nuclear factor kappa B
ng	Nanogram
P38 MAPK	P38 mitogen-activated protein kinases
p70S6K	Ribosomal protein S6 kinase beta-1
pg	Picogram
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PKR	RNA-dependent protein kinase
PLA	Placebo
R	Whole body resistance
RCT	Randomized controlled trial
RDA	Recommended dietary allowances
RET	Resistance exercise training
RF	Rectus femoris
RHEB	Ras homolog enriched in brain
ROS	Reactive oxygen species
SIRT1	Silent information regulator transcript 1

SKF	Skinfolds
SMM	Skeletal muscle mass
STAT3	Signal transducer and activator 3
TBW	Total body water
TCA	Tricarboxylic acid cycle
TDEE	Total daily energy expenditure
TNF-α	Tumor necrosis factor α
UPP	Ubiquitin proteasome pathway
US	Ultrasound
UWW	Underwater weighting
VEGF	Vascular endothelial growth factor
VL	Vastus lateralis
W	Watt
WBPB	Whole-body protein breakdown
WBPS	Whole-body protein synthesis
Xc	Whole body reactance
α-HICA	α-hydroxy-isocaproic acid
α-KIC	α-ketoisocaproate

Abstract

Leucine metabolites β-hydroxy-β-methylbutyrate (calcium, HMB-Ca and free acid, HMB-FA) and α-hydroxyisocaproic acid (α-HICA) have been proposed to enhance performance (muscle power and strength), body composition (muscle thickness, fat-free mass [FFM], fat mass [FM] and bone mineral content [BMC]) and to modulate training-induced hormonal (testosterone, cortisol, insulin-like growth factor-1 [IGF-1] and growth hormone [GH]), inflammatory (tumor necrosis factor α [TNF-α], interleukine 6 [IL6] and high-sensitivity C-reactive protein [hsCRP]) and muscle damage responses (creatine kinase [CK]), in healthy young resistance trained individuals. Additionally, some leucine metabolites have also been proposed to improve functionality and body composition in elderly populations and/or under clinical settings. The present dissertation is comprised of four research studies conducted to further elucidate the effects of these compounds in both young resistance trained men and an older type 1 diabetic individual. Three studies were conducted in 40 young resistance trained men, directly comparing these leucine metabolites with placebo, over 8 weeks of a supervised resistance exercise training, regarding changes in muscle thickness, body composition, several hormones, inflammation and proxy markers of muscle damage. One clinical case study was also conducted in a type 1 diabetic patient to assess the effects of α-HICA on body composition, isometric strength and full hematologic measures, over 120 days without any exercise. No leucine metabolite resulted in any ergogenic effects on any outcome variable in young resistance-trained men, during the 8 weeks of the resistance training, while in the clinical case study a body mass increase was detected due to an increase in trunk FFM. Small increases regarding handgrip strength and bone mineral density were also noted in this clinical case study, albeit of unknown clinical significance. Leucine metabolites are not an effective strategy to improve muscle strength or body composition in young resistance-trained men. More research is warranted regarding α-HICA in diseased populations, particularly to compare different leucine metabolites.

Key-words: β-hydroxy-β-methylbutyrate free acid, β-hydroxy-β-methylbutyrate calcium, α-hydroxyisocaproic acid, body composition, performance.

Resumo

Os metabolitos da leucina (LM) β-hidroxi-β-metilbutirato (cálcico, HMB-Ca e livre, HMB-FA) e ácido hidroxiisocapróico (α-HICA) têm sido propostos na melhoria do desempenho (potência e força muscular), composição corporal (espessuras musculares, massa isenta de gordura [FFM], massa gorda [FM], conteúdo mineral ósseo [BMC]) e modulação hormonal induzida pelo exercício (testosterona, cortisol, factor de crescimento semelhante à insulina [IGF-1] e hormona do crescimento [GH], inflamação (factor de necrose tumoral α [TNF-α], interleucina 6 [IL-6] e proteína C-reactiva de alta sensibilidade [hsCRP]) e marcadores de dano muscular (creatina *cinase* [CK]), em indivíduos jovens, saudáveis e com experiência no treino da força. Adicionalmente, alguns LM também foram propostos na melhoria da mobilidade e composição corporal de populações idosas e/ou em contextos clínicos. A presente dissertação é composta por quatro estudos, conduzidos de forma a fornecer mais evidência em relação aos efeitos destes compostos em homens jovens, treinados e num indivíduo diabético tipo 1. Três estudos foram conduzidos em 40 jovens treinados, comparando directamente estes LM com um placebo, durante 8 semanas de treino da força supervisionado, em relação a alterações na espessura muscular, composição corporal, hormonas, inflamação e dano muscular. Um estudo de caso foi realizado num diabético tipo 1 para avaliar os efeitos do α-HICA na composição corporal, força e parâmetros hematológicos, durante 120 dias, na ausência de exercício. Nenhum LM resultou em quaisquer efeitos ergogénicos, no que diz respeito a qualquer variável estudada, em homens treinados, durante as 8 semanas do protocolo de treino da força, enquanto no estudo de caso, foi detectado um aumento na FFM do tronco. Pequenos incrementos em relação à força de preensão manual e à densidade mineral óssea também foram observados neste estudo de caso, embora de significado clínico desconhecido. Suplementar com metabolitos da leucina não é uma estratégia eficaz para melhorar a força muscular ou a composição corporal em homens jovens treinados. São necessários mais estudos em relação ao α-HICA, em populações clínicas, que comparem de forma directa os diversos metabolitos da leucina.

Palavras-chave: β-hidroxi-β-metilbutirato livre, β-hidroxi-β-metilbutirato cálcico, ácido hidroxiisocapróico, composição corporal, desempenho.

CHAPTER 1

General Introduction

1.1 Dissertation Structure

The study of leucine metabolites on performance, body composition and several biochemical markers has been thoroughly investigated for over two decades. β-hydroxy-β-methylbutyrate (HMB), the deaminated and decarboxylated form of leucine, has been the most studied compound with over 50 peer reviewed published publications. The evidence pertaining leucic acid or α-hydroxy-isocaproic acid (α-HICA) is scant, with only one research study being published to date. The present dissertation, entitled "The effects of leucine metabolites on performance, body composition and biochemical markers of muscle damage and inflamamation", aimed to clarify whether supplementing with these leucine metabolites may, in fact, be an effective strategy to enhance performance, improve body composition and ameliorate biochemical markers of muscle damage and inflammation.

In order to contextualize this investigation, that led to the publication of four research studies in peer-review journals with an established ISI Impact Factor or SCImago journal rank, a literature review was performed (Chapter 2), and a general discussion (Chapter 8), providing a summary and some insights regarding the main findings from these studies (Chapters 4-7). This dissertation is organized as follows:

Chapter 2 includes an extensive literature review regarding leucine and its metabolism: action on muscle and energy metabolism, glucose homeostasis, immunomodulation and inflammation. A brief review on its effects in humans was also performed. Additionally HMB was also reviewed pertaining its metabolism, differences in bioavailability between different forms of HMB (HMB-Ca, HMB-FA), safety, duration of supplementation, dose and timing. Proposed mechanisms of action were also reviewed, as well as HMB's actions in humans regarding improvements in body composition, performance and biochemical markers (both in young and elderly subjects). Bearing this in mind, we reviewed the current literature regarding leucine metabolites (HMB and α-HICA), along with their main limitations and recommended future prospects.

The four studies included a generalized description of the methods used in each particular investigation, however a detailed and more specific description of all methodologies used is described in **Chapter 3**.

Chapters 4 to 7 correspond to the four studies that were conducted to answer the research goals that were described in Chapter 2.

Chapter 8 corresponds to a general discussion, further discussing the main findings, limitations and futures prospects from the research from these four studies (chapters 4-7). General conclusions, bearing in mind the main findings of this investigation, were crafted at the end of this section.

The bibliographic references were presented in the end of each section, adopting a number format.

1.2 List of articles and conference abstracts as first author

As a result of the complementary work that occurred as a significant part of the doctoral research program, publications in international journals and communications (oral/poster) in international congresses were made as first author:

PEER-REVIEWED ARTICLES PUBLISHED, IN PRESS OR SUBMITTED FROM THE DISSERTATION:

Teixeira FJ, Matias CN, Monteiro CP, Valamatos MJ, Reis JF, Tavares F, Batista A, Domingos C, Alves F, Sardinha LB, Phillips SM. **Leucine Metabolites Do Not Enhance Training-induced Performance or Muscle Thickness**. Medicine & Science in Sports & Exercise. 2019;51(1):56-64.

Teixeira FJ, Matias CN, Monteiro CP, Valamatos MJ, Reis JF, Batista A, Oliveira AC, Alves F, Sardinha LB, Phillips SM. **No effect of HMB or α-HICA supplementation on training-induced changes in body composition**. European Journal of Sport Science. 2018:1-9.

Teixeira FJ, Matias CN, Monteiro CP, Valamatos MJ, Reis JF, Morton RW, Alves F, Sardinha LB, Phillips SM. **Leucine metabolites do not attenuate training induced**

inflammation in young resistance trained men. Journal of Sport Sciences (under review).

Teixeira FJ, Matias CN, Monteiro CP, Kones R. **Effects of Alpha-hydroxy-isocaproic acid upon Body Composition in a Type I Diabetic Patient with Muscle Atrophy - A Case Study**. The Yale journal of biology and medicine. 2018;91(2):161-71.

ABSTRACTS AND POSTERS THAT ARE RELATED WITH THE DISSERTATION:

Teixeira FJ, Matias CN, Monteiro CP, Valamatos MJ, Reis JF, Tavares F, Batista A, Domingos C, Alves F, Sardinha LB, Phillips SM. **No effect of HMB or α-HICA on training-induced changes in performance or body composition**. World Congress on the Basic Science of Muscle Hypertrophy and Atrophy - American College of Sports Medicine; Minneapolis 2018.

Teixeira FJ, Matias CN, Monteiro CP, Valamatos MJ, Reis JF, Tavares F, Batista A, Domingos C, Alves F, Sardinha LB, Phillips SM. **No Effect Of Hmb Or α-hica On Training-induced Changes In Performance Or Body Composition: 503 Board #1**. Medicine & Science in Sports & Exercise. 2018;50(5S).

OTHER PEER-REVIEWED ARTICLES PUBLISHED DURING THE COMPLETION OF THE DISSERTATION:

Batista A, Monteiro CP, Borrego R, Matias CN, **Teixeira FJ**, Valamatos MJ, Oliveira AC, Reis JF, Mendes L, Sardinha LB. **Association between whey protein, regional fat mass, and strength in resistance-trained men: a cross-sectional study**. Applied Physiology Nutrition, and Metabolism. 2019;44(1):7-12.

Santos HO, **Teixeira FJ**. **Use of medicinal doses of zinc as a safe and efficient coadjutant in the treatment of male hypogonadism**. The Aging Male: The Official

Journal of The International Society for the Study of Aging Male. 2019;15:1.10. doi: 10.1080/13685538.2019.1573220

Santos HO, Howell S, **Teixeira FJ**. **Beyond tribulus (Tribulus terrestris L.): The effects of phytotherapics on testosterone, sperm and prostate parameters**. Journal of Ethnopharmacology. 2019;pii:S0378-8741. doi: 10.1016/j.jep.2019.02.033

CHAPTER 2

Literature Review

Amino acids are more than precursors for *de novo* protein synthesis, in fact they also exert important roles in molecular signalling and regulation of several metabolic processes such as: energy regulation, insulin homeostasis and immunomodulation (1). Moreover, leucine has been proposed as a potent activator of protein synthesis and simultaneously as an inhibitor of muscle proteolysis, which may lead to an increased expression of the anabolic phenotype (2). Leucine seems to regulate an important evolutionary pool of kinases, referred as mechanistic target of rapamycin complex or mTORC1, with the former purportedly regulating protein synthesis (3). How exactly leucine may influence muscle protein synthesis and proteolysis is presently unclear, although Dodd & Tee (2) have proposed several mechanisms.

It should however be noted, that research *in vivo* regarding body composition and performance with leucine has exerted equivocal results (2). Conversely, the supplementation with leucine metabolites, namely β-hydroxy-β-methylbutyrate (HMB) and leucic acid (α-HICA) has displayed noteworthy results (4-7). Bearing this in mind, the evidence regarding leucine and its metabolites: HMB and α-HICA, will be reviewed during this chapter.

2.1 Leucine

Leucine ($C_6H_{13}NO_2$), also known as 2-amino-4-methylpentanoic acid is an indispensable amino acid with a primary role in haemoglobin formation (8). Due to its arrangement of carbon atoms, leucine is one of the three branched-chain amino acids (BCAAs), with the other two being valine and isoleucine. This amino acid is present in all proteins (9) specially in high-quality foods (10) displaying a singular signalling role in several types of cells (11). Main dietary sources of leucine are animal foods, specially dairy, with vegetable sources as maize displaying also significant concentrations of this amino acid (12).

Leucine is important in several biochemical pathways, that mediate both protein synthesis and glucose homeostasis, activating molecules like phosphoinositide 3-kinase/protein kinase B and mTOR (13, 14). Unlike other indispensable amino acids, BCAAs are initially metabolized in extrahepatic tissues by branched-chain amino acid aminotransferases (BCAT) and branched-chain α-keto acid dehydrogenase enzyme

complex (BCKDC) (15). Additionally, leucine may improve lipid flux into the muscle tissue, further supporting the high demand of energy required for protein synthesis (16).

Metabolism

Leucine is transported by the system *L* transporters (LAT) which include several sodium independent isoforms (1 to 4). Leucine transport is dependent on glutamine which is carried into the cells by the Na^+-dependent alanine-serine-cysteine-preferring transporter 2 (ASCT2) and then is transported outside the cells by LAT1. The former uses intracellular glutamine as an efflux substrate to uptake extracellular leucine into the cells (17, 18). Since leucine may also increase gene expression of some amino acid transporters (i.e. ASCT2) it is reasonable that it may also regulate the transport of other neutral and cationic amino acids (19).

Once inside the cells, leucine is metabolized by reversible transamination to form α-ketoisocaproate (α-KIC) resulting in the production of glutamate by the enzyme BCAT (20). The later enzyme is highly expressed in the myocytes (21) but not in the liver (22, 23). This biochemical feature explains, at least partially, why leucine is primarily oxidized in the muscle and not in the liver.

The oxidation of leucine in the skeletal muscle depends on BCKDC, which presents a low level of activity at rest (5-8%) but may be further stimulated by exercise up to 20-25% (24, 25). This complex is activated at the onset of exercise by dephosphorylation, in response to high concentrations BCAAs inside the muscle fiber, low levels of glycogen, low pH and also a decreased ATP/ADP ratio (26). Thus, an increase in leucine oxidation is only detected when either abundant exogenous amounts are provided, muscle glycogen stores significantly decrease or the levels of energy in the muscle fibre drop (27). A decrease in glycogen degradation in both the muscle and the liver are noted, after leucine supplementation (28, 29).

One of the resulting products of leucine transamination is glutamate, which may either be amidinated with ammonia to form glutamine or react with pyruvate forming alanine and oxoglutarate (30). Eventually, all BCAAs may be transaminated and decarboxylated into end products that enter the tricarboxylic acid cycle (TCA) (figure 1).

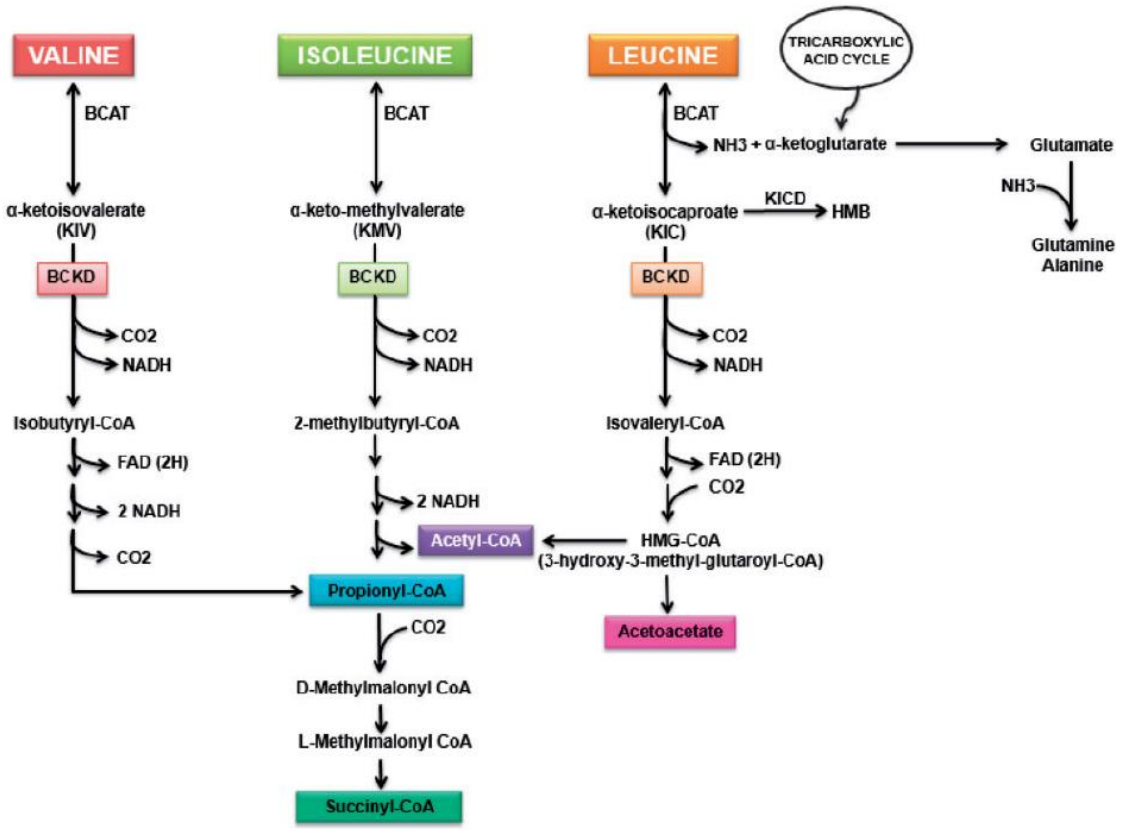

Figure 1. Simplified main steps of BCAAs catabolism adapted from (18). In the first step a conversion into their keto acids occurs in extrahepatic tissues (muscle), while in the liver they are irreversibly decarboxylated (second step). In the third and last step their downstream metabolites will enter into the adenosine triphosphate synthesis process.

The tissue supply of leucine depends on both exogenous (dietary) or endogenous (protein breakdown) supply (31). Since leucine can surpass transamination processes in the liver, feeding provides a sharp rise in plasma concentrations (32). After leucine is converted into α-KIC, this metabolite is further oxidized or re-synthesised back to leucine by extrahepatic tissues. At the muscle site, leucine is mainly used for protein synthesis or interconverted to alanine and glutamine (33).

After being formed from leucine by reversible transamination, α-KIC undergoes a second step of irreversible oxidative decarboxylation by BCKDC in the liver mitochondrion to form isovaleryl CoA (IVA-CoA). Approximately 90% of all α-KIC is oxidized to IVA-CoA and downstream metabolites acetoacetate and acetyl-CoA. At the liver cytosol, the remaining α-KIC is converted to β-hydroxy-β-methylbutyrate (HMB) by α-KIC dioxygenase (KICD) (figure 2) (31). Thus, conversion into HMB is

simultaneously a route of elimination by the kidney (34-36) and a pathway for cholesterol synthesis – via β-Hydroxy-β-methylglutaryl-CoA (HMG-CoA) (37).

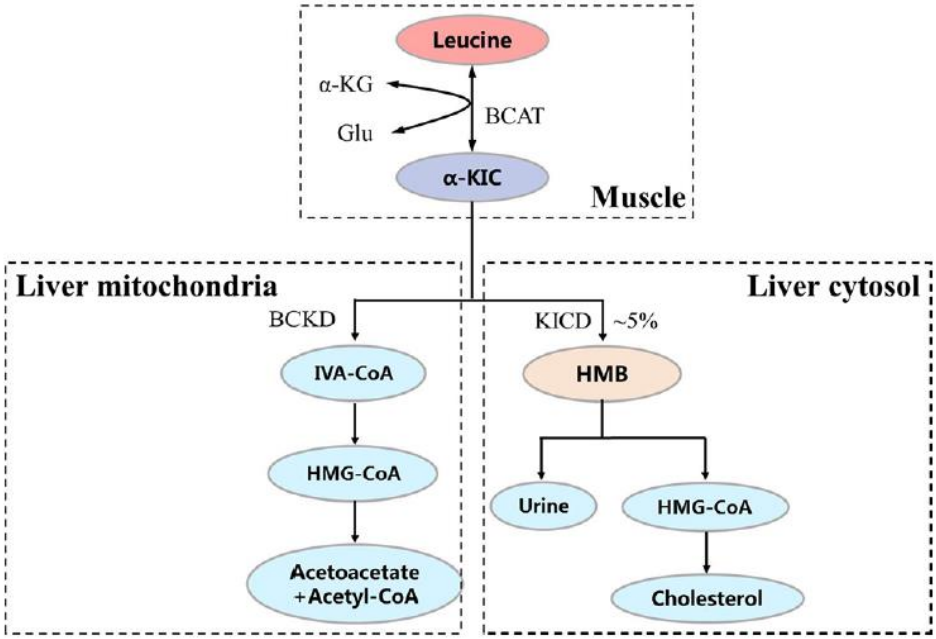

Figure 2. Leucine metabolism in animals adapted from (38).

Abbreviations: α-KG: α-ketoglutarate; α-KIC: α-ketoisocaproate; BCAT: Branched-chain amino acid aminotransferases; BCKD: Branched-chain α-keto acid dehydrogenase; Glu: glutamine; HMG-CoA: β-Hydroxy-β-methylglutaryl-CoA; IVA-CoA: Isovaleryl CoA; KICD: α-KIC dioxygenase.

Many of the anticatabolic and anabolic actions of leucine have been attributed to the leucine metabolites α-KIC, HMB and α-HICA (6, 39). However the amount required to induce an effect cannot be consumed within a normal diet (40). Approximately 80% of leucine is used for protein synthesis with the remainder being converted to α-KIC and only 5% to HMB (38). Thus, within a normal diet, only 0.2-0.4 g HMB.day^{-1} is synthesized from leucine in a 70 kg individual (36, 41). Since the lower amount that exerted performance and body composition enhancement was 1.5 g.day^{-1} (42), this amount is deemed insufficient. To attain such a high intake of HMB, ≈60 g of leucine would have to be consumed daily, which is beyond the intake of a regular diet (43) and above the current recommended dietary allowances ([RDA] - 42 mg/kg.day^{-1}) (39). Although other BCAAs (isoleucine and valine) have also been proposed to induce similar effects, the current body of evidence does not support a similar metabolic action for these amino acids (37).

Action on muscle protein synthesis

Leucine actions upon muscle protein synthesis (MPS) have been researched for over four decades (44-46), yet the involved mechanisms remain unclear to date. It has been proposed that its downstream metabolites are, at least in part, responsible for this anabolic action (47-49). However, earlier studies proposed that leucine could influence MPS (50) without being converted to α-KIC (51). Consequently, leucine was thoroughly investigated regarding MPS (52-54).

Leucine exerts a double action, both as substrate and as a signalling molecule for the initiation of MPS (55, 56). Several reviews have proposed numerous possible mechanisms, albeit a consensus has not been reached (10, 38, 57-60). As proposed by Duan et al (38), it is likely that after an increase in intracellular concentrations of leucine, Ras-related guanosine triphosphatases (61), human vacuolar sorting protein-34 (62) and mitogen activated protein kinase 3 (61) are activated causing the translocation of mTORC1 to the surface of late endosomes and lysosomes, where it is directly activated by Ras homolog enriched in brain (RHEB) at the lysosome's surface. Further mTORC1 activation will lead to the downstream phosphorylation and activation of proteins that will regulate both translation initiation and elongation steps (figure 3). However, although leucine might increase insulin secretion via phosphoinositide 3-kinase (PI3K) activity, this does not seem important regarding mTORC1 stimulation (63, 64). Any insulin release by leucine, or its metabolites, is only permissive towards the muscle protein synthetic response and not the main driving factor for MPS to occur (38).

More recently this concept has been challenged, with some leucine metabolites – HMB and α-KIC – being touted has possessing direct anabolic properties and being responsible for the anabolic actions of leucine (20). Recent research, has further proposed a novel form of HMB (HMB free acid) and α-HICA to exert both anabolic and anti-catabolic properties (5-7). Please refer to chapter 2.2 for a more detailed discussion on these leucine metabolites.

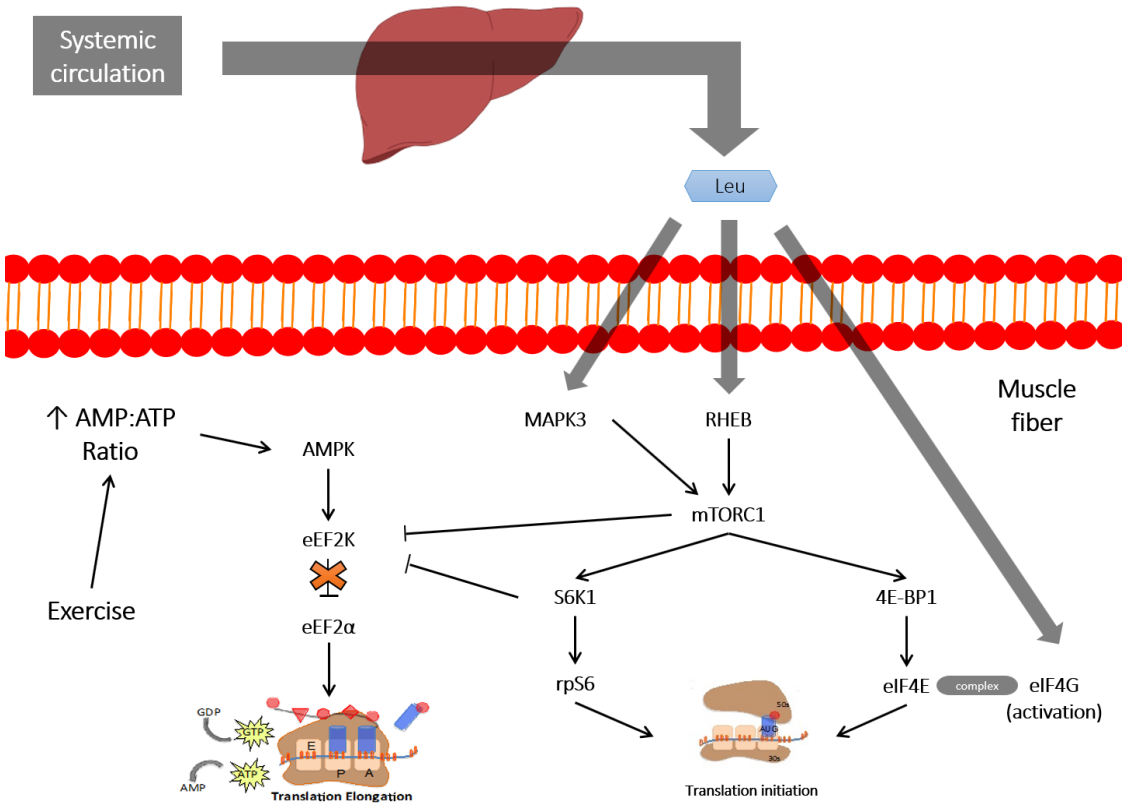

Figure 3. Leucine pathways that may influence MPS (65, 66).

Action on muscle protein degradation

When low levels of amino acids or energy are sensed by the cells, MPS is hindered while the muscle protein breakdown (MPB) response is enhanced (degradation pathway) (67). Several mechanisms have been proposed, mainly from animal or *in vitro* studies, regarding the leucine effect on proteolysis although no consensus seems to exist so far. Some authors have proposed mTOR signalling, excluding an upstream kinase PI3K activation (68); while others have suggested the involvement of both PI3K and protein kinase C (PKC) (69).

It should also be noted, that the concentration of leucine used in some of these *in vitro* studies (10 mM) is ≈ 50 fold the plasma concentration found *in vivo*, therefore it is highly questionable that these levels may be attainable through dietary intake (38). Albeit some studies have suggested that protein inhibition with leucine might be mediated by

some of its metabolites (22,84,85), in particular HMB (70, 71), more recent research has displayed equivocal evidence (72). Definitely more research is commended to provide further mechanistical insights, regarding the suppression of proteolysis by leucine and also pertaining its downstream metabolites.

Energy metabolism

Leucine may also influence energy metabolism, since protein synthesis and energy are closely related through mTORC1 and AMP-activated protein kinase (AMPK) (57). In fact, leucine goes far beyond protein synthesis, since downstream regulatory proteins from the mTORC pool of kinases also promote mitochondrial biogenesis, further enhancing cellular respiration and energy partitioning (16, 73). It has been proposed, from observations in animal research, that by ameliorating cellular metabolism both fatty acid oxidation and lean tissue gains may be enhanced (57).

Some research has suggested that some positive effects regarding mitochondrial biogenesis and fatty acid oxidation, leading to an increase in energy expenditure, are the result of AMPK and silent information regulator transcript 1 (SIRT1) activation by leucine (73). Whether leucine mediates energy metabolism directly or through its metabolites, is unclear to date (38). Several proteins, that are under the influence of leucine or its metabolites, are involved in the regulation of both oxidative phosphorylation, glycolysis activation and fatty acid β-oxidation (74), with also the nitric oxide pathway probably being involved (75, 76).

Glucose homeostasis

Insulin may translocate the glucose transporter 4 to the plasma membrane of both muscle cells and adipocytes promoting glucose uptake (77). This process is mediated by intracellular signaling involving mTOR and PI3K. It has been shown that leucine may improve satiety and glucose metabolism using complex peripheral and central pathways (32, 53). Unlike insulin, leucine may activate protein kinase C and not B, thus promoting glucose uptake by a different pathway (78). Moreover, leucine may induce glucose uptake, for short periods of time, in a dose-dependent manner via mTORC1 and 2 (79).

It seems that this short-term glucose uptake (up to 45 min) (78), is likely due to the negative feedback resulting from the continuous mTOR stimulation (80). This small effect, in glucose uptake, is likely irrelevant, since research measuring *in vivo* net glucose transport, has failed to detect any effect of this amino acid (81). Albeit the stimulation of mTOR is deemed important in the insulin signaling process, an excessive activation may conversely lead to impaired insulin sensitivity (79).

Leucine may also stimulate insulin secretion from pancreatic β cells via two different mechanisms (82). It may serve as metabolic fuel for β cells or exert its action as an allosteric activator of glutamate dehydrogenase (GDH) (83), which may in turn promote insulin release from the pancreatic islets (84). It seems that both leucine or its transaminated product α-KIC, may act upon insulin secretion by direct inhibition of the ATP-regulated potassium channels (85). Both compounds regulate these channels, by increasing Ca^{2+} in the β cell's cytosol, which would ultimately lead to the exocytosis of insulin secretory granules, probably via both protein phosphorylation and acylation mechanisms (86, 87). Other described mechanisms that may regulate β cell metabolism and insulin secretion, with leucine, are related with an increase in protein synthesis and gene expression (82).

Summarizing, leucine is able to acutely stimulate insulin secretion in β-cells, via ATP production and ATP-regulated potassium channels and also GDH activation. Moreover, acute effects and chronic effects have been described via increased protein synthesis and gene expression in β cells. It should however be noted, that most of the aforementioned mechanisms are the result of *in vitro* studies, with *in vivo* research failing to detect meaningful effects of leucine in glucose transport (81).

Immunomodulatory role

Few studies have investigated the role of leucine pertaining inflammation and immune cells (18). Immune cells have indeed high BCAT and BCKD activity levels, using also leucine among other BCAAs throughout the S phase of the cell cycle (88). During enhanced mitogenesis, it has been estimated that the BCAAs transport may be increased by ≈ 300%, while transamination and oxidation are increased by ≈ 200% and ≈ 130%, respectively (89).

When T cells (cluster of differentiation 4 [$CD4^+$]) are activated, their requirements for amino acids increase. This is likely related with augmented protein synthesis requirements, which are mandatory for proper T cell function (18) and proliferation. The activated cells decrease the decarboxylation of pyruvate, yielding higher lactate levels that may supply nicotinamide adenine dinucleotide (NAD^+). Indeed, *in vitro* studies have shown, that BCAAs may activate T cells (90). Furthermore, a BCAT increase in immune cells, is positively correlated with both leucine transamination and mTOR activation, with the former being a critical regulator of T cell activation, differentiation, and metabolism (90). Albeit leucine has been implicated as an important amino acid pertaining lymphocyte activation, other BCAAs may also play an important role in this regard (91).

Although animal studies have shown promising results regarding leucine and its immunomodulatory role (92-95), few studies have been performed in humans (96). It seems indisputable, that leucine is both oxidized by immune cells and incorporated as a precursor for the synthesis of new cells (97). However, albeit promising, the effect of leucine or BCAAs regarding a possible positive role in immunity, should be further investigated in humans, definitely requiring further confirmatory research.

Role in inflammation

Inflammation plays an important role in tissue regeneration and protection (98) with the translocation of nuclear factor kappa B (NF-κB) to the cell nucleus long being implicated in the cell's inflammatory response. Mechanisms that allow for this translocation to occur seem related with a plethora of stimuli, i.e. increases in inflammatory markers (Tumor necrosis factor α [TNF-α] and interleukin 1 [IL-1]), lipopolysaccharides (LPS), heat shock proteins (HSP) and several T-cell activators (99). After being translocated, NF-κB will upregulate the expression of several immune-mediated factors at the cell nucleus, namely cyclooxygenase 2 (COX-2) and several inflammatory cytokines, that will ultimately aid on the eradication of several infectious agents (100, 101). Albeit inflammation is necessary for tissue regeneration and protection, overstimulating NF-κB may lead to life-threatening outcomes (18). More recently, it has

been suggested that NF-κB inhibition may inhibit apoptosis and reduce vascular endothelial growth factor (VEGF) (102-104).

Moreover, mTOR and NF-κB seem related (105). Indeed, the stimulus of NF-κB under certain oncological contexts, seems to contribute to cell proliferation, growth and angiogenesis (albeit this seems to be dependent on other factors like TNF-α) (106). Although inflammation may increase the mTOR response, it may paradoxically impair protein synthesis (107) and thus hinder the anabolic response to amino acids, especially leucine (108). Albeit a theoretical advantage could exist by supplementing with leucine in inflammatory diseases to hinder protein degradation, animal studies do not show advantages regarding supplementation, even with high doses of leucine or other BCAAs (107). This is likely due to the reduction of intramuscular glutamine from inflammatory diseases that may impair leucine transport to the muscle, since the transport of leucine is dependent on the intracellular concentration of glutamine (109, 110).

Few studies have investigated BCAAs or leucine regarding inflammation in humans. Recently, Nicastro et al. (111) evaluated both the supplementation with BCAAs, leucine or placebo (PLA) 15, 30, 60, 90 and 120 minutes after intake, in the absence of any exercise protocol. The authors reported no differences between groups insofar as TNF-α is concerned, however serum interleukin 6 (IL-6) decreased, while interleukin 10 (IL-10) significantly increased 60 min after, in the leucine group only. However, in this crossover controlled trial (n=8 participants) volunteers were refrained to perform resistance exercise 24 h prior to the supplementation protocol and blood collection, which precludes any extrapolation to a post-exercise context. Although statistically significant, these actions from leucine in the absence of an inflammatory stimulus, seem of minor importance regarding diseased populations or athletes.

Human research on performance and body composition

Leucine ingestion positively correlates with MPS in middle aged men, with a strong direct correlation being reported between peak plasma leucine concentrations and the postprandial muscle leucine accretion as evaluated by the postprandial muscle protein fractional synthetic rate (FSR) (112). Bearing this in mind, when aiming to induce maximal hypertrophic outcomes, some protein rich foodstuffs have been purported as

superior due to a higher leucine content and a faster digestion rate (113). Albeit leucine has been deemed important to initiate MPS (114), other essential amino acids are also necessary (115). Some research has suggested that administering leucine alone might rescue the FSR, when lower amounts of essential amino acids (EAAs) are present (116). Conversely, administering leucine with isoleucine and valine (other BCAAs), with limited amounts of other EAAs, might reduce the MPS response likely due to competition for the same gut transporter, which may decrease plasma leucine concentrations (115). In fact, a recent review suggests an absolute intake of 20-40 g of protein/meal (10-12 g of EAAs, 1-3 g of leucine) or 0.25 g/kg in young individuals, with higher doses being recommended for elderly individuals (≈40 g/meal), to maximize the MPS (117).

Few studies have investigated the effects of leucine in young healthy individuals. Some research has shown that leucine supplementation may increase strength, not improving body composition (i.e. not decreasing fat mass [FM] nor increasing fat free mass [FFM]) (118), with other research studies further confirming this evidence while failing to show improvements in body composition or muscle strength gains (119). A study by Crowe et al. (120), in young competitive canoeists, investigated the effects of 45 $mg.kg^{-1}.d^{-1}$ of leucine or placebo for six weeks. Although leucine concentrations increased in the blood and some improvements in endurance performance were reported, no effects on body mass (BM) or percentage body fat were found between groups. This was in agreement with a review by Balage et al. (121), stating that leucine-rich amino acid mixtures or proteins (providing sufficient EAAs) appear more efficient than leucine alone to improve muscle mass and performance, suggesting that the efficacy of leucine depends on the presence of other EAAs. Additionally, a review by Yao et al. (122) has proposed leucine as a promising nutritional tool to fight obesity, with several mechanisms being proposed (figure 4). However, most of the described pathways in this review are the result of animal studies, with few research studies being performed in humans (only 2 out of 14 studies).

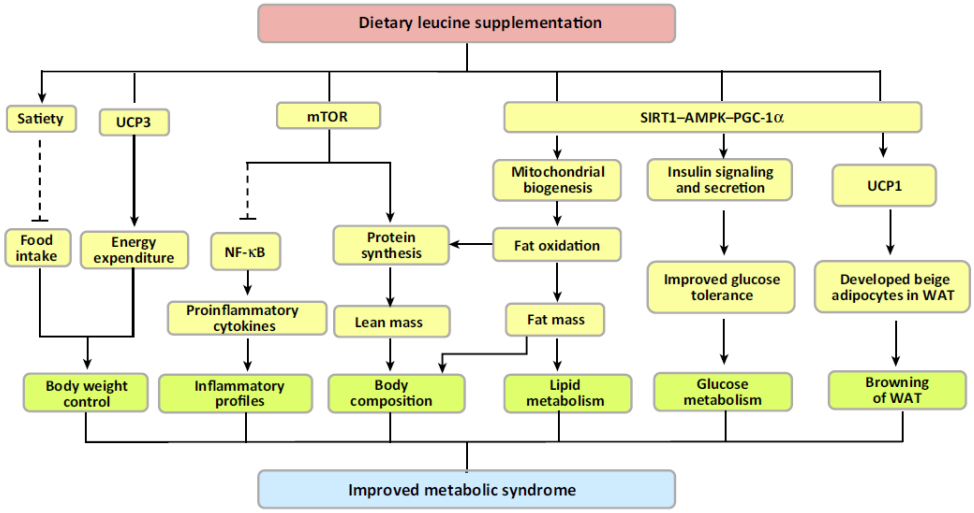

Figure 4. Proposed mechanisms for a beneficial effect of leucine on obesity/metabolic syndrome (122).

Insofar as maintaining FFM, while undergoing food restriction, few studies have been performed in humans (younger or older individuals) with the majority being performed in animal models (123-127). Albeit some reviews have suggested an important role of leucine in preventing FFM losses, most seem unanimous on claiming that this amino acid requires other essential amino acids to be effective in preventing lean mass losses (1, 121). The current body of evidence pertaining leucine supplementation (alone), to prevent FFM while in caloric restriction is scant, with more human research being mandatory at this point.

Studies regarding leucine alone in elderly populations are also limited. Interestingly Ispoglou et al. (128) showed benefits with 6 g of leucine daily in addition to other essential amino acids. Elderly participants (n=36, 65-75 years old) displayed improvements regarding functional status and FFM, after 3 months of supplementation with a mixture of essential amino acids enriched with leucine. The authors proposed that leucine and BCAAs might be interesting to improve functionality and lean mass retention in older individuals, albeit reinforcing that leucine alone would not offer any additional benefits and that longer term studies are required. Some reviews have also suggested that leucine supplementation might be a promising strategy to prevent muscle mass loses with aging and to improve postprandial glycemic control in patients with type 2 diabetes (129). Still, authors state that there is insufficient evidence to recommend dietary supplementation with leucine to augment muscle mass, strength or improve glycemic

control. In fact, not all research supports a positive effect of leucine supplementation on muscle mass or strength, at least in healthy elderly men (130).

Although safe up to 1250 $mg.kg^{-1}.d^{-1}$ (131), studies performed in elderly populations have similar outcomes to their younger counterparts. Moreover, adding leucine to lower amounts of essential amino acids may pose as an effective strategy to stimulate muscle anabolism both at rest and after exercise (132). Murphy et al. (133) have further confirmed, that leucine co-ingestion with lower daily protein intakes ($0.8\ g.kg^{-1}.d^{-1}$) will augment muscle protein synthesis. Since older individuals do not typically ingest sufficient protein to optimally stimulate MPS ($0.4\ g.kg^{-1}.meal^{-1}$) due to age-related issues (134-136) or as imposed to slow the progression of certain chronic diseases (137), leucine supplementation along with meals may be an effective strategy to attenuate muscle loss. Additionally, combining leucine with other dietary supplements (i.e. vitamin D or whey), might also be promising to prevent sarcopenia in older persons and has been proposed in a recent review (138).

Altogether, it is plausible that leucine rich protein supplements might exert beneficial effects on body weight, body mass index and FFM in older persons prone to sarcopenia, but not muscle strength (albeit an elevated heterogenicity was detected between trials in the meta-analysis performed by Komar et al.) (139). A more recent meta-analysis by Xu et al. (140) confirmed that leucine significantly increases MPS, albeit failing to show a significant effect on FFM. Increases in MPS may be important to prevent skeletal muscle losses with aging, however supplementing with leucine alone might not be the best strategy, since to maximize MPS all essential amino acids are required (1).

2.2 Leucine metabolites

2.2.1 β-hydroxy-β-methylbutyrate (HMB)

For decades, leucine metabolites have been thoroughly studied regarding their anabolic or anticatabolic potential (141). Several studies have been performed towards one particular metabolite, HMB (the deaminated and decarboxylated metabolite of

leucine). This was the result of an early study by Nissen et al. (42) that displayed interesting results regarding both body composition and performance outcomes and shifted the focus towards the inhibition of leucine transamination to spare muscle protein, via HMB (48). Since metabolites from other BCAAs lacked the same effect, the focus was placed towards leucine and its derivatives (37). As previously stated, nearly 60 g of leucine is required to produce 3 g of HMB (49). Such high amount, definitely requires a supplementation strategy with HMB, to elicit higher protein synthesis rates and minimize protein breakdown (142).

Several research studies have been performed to date with HMB (both HMB-Ca and HMB-FA), additionally several systematic reviews/meta-analysis (41, 143-150) and narrative reviews (43, 151-164) were also performed. Currently, it has been estimated that ≈2% of college athletes consume HMB (165). Although a considerable amount of evidence is available, some aspects of HMB remain controversial, with more research in some areas being commended.

METABOLISM

HMB is a natural biologically active compound present in many foods such as: catfish, alfalfa, asparagus, avocado, cauliflower and grapefruit (166). In animals and humans, HMB is mostly produced from leucine (167) in the liver from an extrahepatically derived metabolite (168). After leucine is converted to α-KIC in the muscle by BCAT, this α-ketoacid undergoes two pathways in the liver: a) the conversion to HMB in the cytosol by KICD (169) or b) the conversion to IVA-CoA by BCKDC in the mitochondria (31, 166). The main route for HMB synthesis is via KICD, albeit the enzyme enol-CoA hydrase (ECoAH) might also convert β-methyl-crotonyl-CoA (MC-CoA) produced from IVA-CoA to HMB when biotin is deficient. HMB undergoes conversion to HMG-CoA via a CO_2 dependent pathway, being ultimately converted to either cholesterol via HMG-CoA reductase (170) or acetoacetyl-CoA via HMG-CoA synthase (figure 5) (43). It seems undisputable, at this point, that HMB is in fact a precursor of cholesterol (171, 172).

When following the biochemical pathway of α-KIC to IVA-CoA via BCKDC, HMG-CoA will also be formed (figure 5). Under normal conditions most α-KIC is converted to IVA-CoA, with only 5% being converted to HMB (36, 49). Nissen and

Abumrad (173) have previously described that the main purpose of HMB is the conversion to HMG-CoA in the liver, to provide substrate for cholesterol synthesis. Bearing this in mind, it has been proposed that HMB supplementation might be important to assure proper cellular function, working as a substrate for HMG-CoA synthesis (43). Since the muscle seems to rely on *de novo* synthesis of cholesterol and the former is important for proper cell function, the role of HMB has been highlighted in this regard, albeit dependent on the degree of cell damage (43).

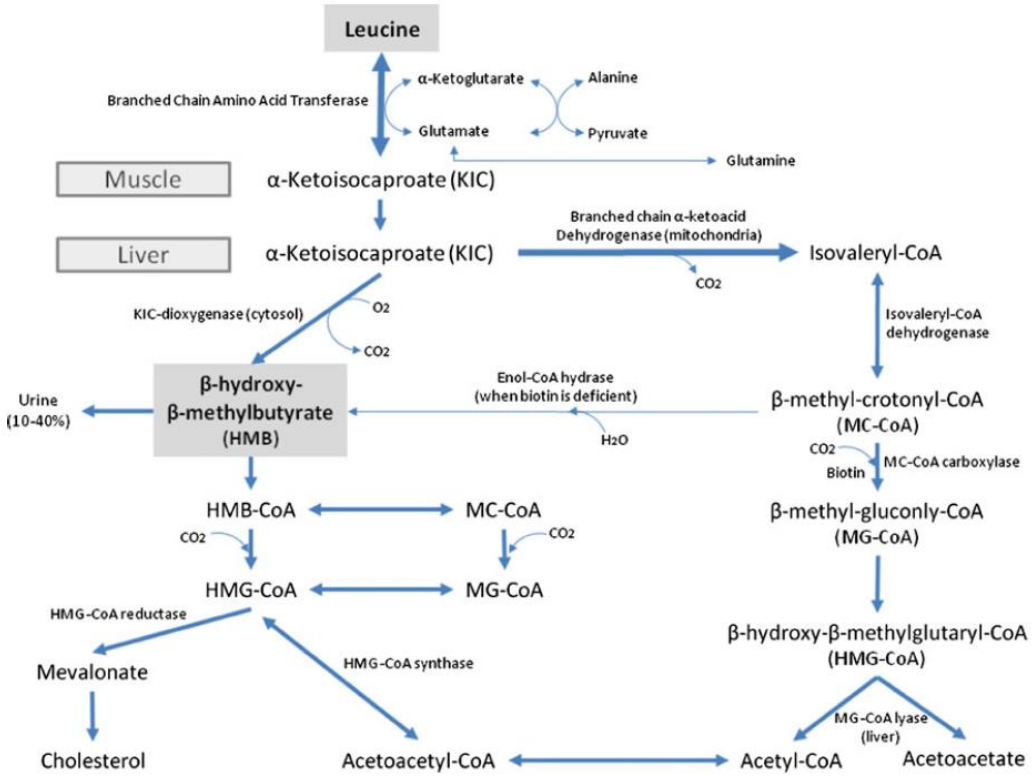

Figure 5. HMB metabolism adapted from Nissen and Abumrad (31) and Zanchi et al. (43).

BIOAVAILABILITY BETWEEN DIFFERENT COMMERCIAL FORMS OF HMB

Since it has been commercially available, HMB has been available in the mono-hydrated calcium salt form (HMB-Ca) with the following empirical formula: $Ca(HMB)_2 \cdot H_2O$ (151). Several factors have been described to influence its rate and magnitude of appearance in the blood. The amount of HMB consumed and the co-ingestion with other nutrients seems to influence the HMB kinetics (174). For example it has been well documented that one g of HMB-Ca will peak HMB blood levels 120 min after ingestion,

while 3 g may speed the absorption kinetics to only 60 min, leading to higher plasma concentrations (487 vs 120 nmol/mL) (174).

It should also be noted that this faster and higher increase in the blood with the 3 g dose, will lead inevitably to higher HMB losses in urine (+14%) (174). Furthermore, adding glucose to HMB may also influence the rate and magnitude of its appearance, with 75 g of glucose delaying peak HMB levels in plasma by one hour and significantly lowering peak levels in the blood (352 vs 487 nmol/mL) (151). Thus, it was hypothesized that glucose might have both slowed gastric emptying and increased HMB clearance from circulation (151).

More recently a new form of HMB has been made commercially available, the free acid form of HMB (HMB-FA) (175). This type of HMB was associated with a gel containing a buffer (potassium carbonate – K_2CO_3) to raise pH to 4.5, which was though to stabilize HMB not hindering the absorption kinetics. In the initial commercially available form, the addition of Ca to HMB was thought to dissociate swiftly (15 min) in the gut and increase its solubility (176). However Fuller et al. (175, 177) showed that when comparing equivalent amounts of HMB from both HMB-Ca and HMB-FA, the former resulted in a two fold increase in peak plasma levels in just one fourth of the time (30 min vs. 120 min – figure 6) (175).

Additionally, the area under the curve analysis over three hours of ingestion was ≈97% higher with HMB-FA. The half-life time of both forms was similar, with a slightly higher time being reported for HMB-FA (3 h vs. 2,5 h) (175). Moreover, albeit HMB-FA leads to greater plasma concentrations, urinary losses were not different between both forms. Interestingly, the tissue uptake and utilization was also 25% higher with the free acid form, which was seen as promising towards amplifying HMB's action within the cell. Albeit, some issues were raised towards the HMB-FA bioavailability in rats (178), more recent research in humans (177) confirmed the previous findings (175), towards a superior bioavailability of HMB-FA in humans, with better bioavailability through liquid-filled caps when comparing with gel format.

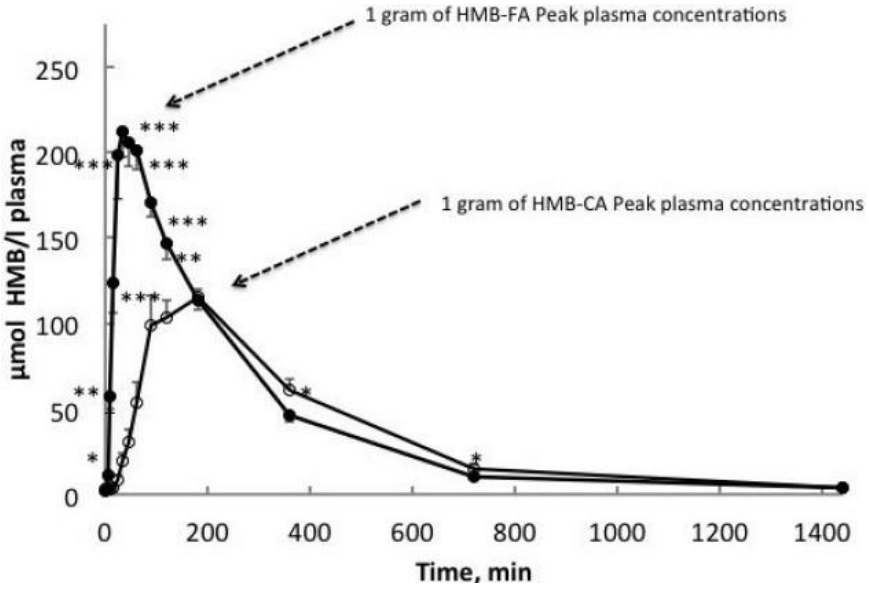

Figure 6. Different absorption kinetics after 1 g of either HMB-Ca or HMB-FA (151).

SAFETY

HMB has been thoroughly studied in humans (31, 172, 179, 180) and deemed to be safe. Human research has showed that 6 g of HMB-Ca daily for one month had no effect on cholesterol, haemoglobin, white blood cells, blood glucose, and liver and kidney function (181). Meta-analysis have also confirmed the high safety profile of HMB in humans (both young, old, diseased or healthy individuals) even when supplemented with amino acids (41, 172, 180). Furthermore, Baier et al. also confirmed the safety profile of HMB (2-3 g daily) for a longer period of time (one year) in elderly individuals, with no adverse effects being reported in blood or urine markers of hepatic or renal function (179). Also no detrimental effects were found towards blood lipids. The longer study performed to date with HMB-Ca, showed no adverse effects in an elderly population, consuming 3 g daily for 24 weeks (182).

Pertaining HMB-FA, data confirms its safety in humans, at doses of 402 and 459 mg/kg bodyweight.day^{-1} for men and women, respectively (183). Human research up to 12 weeks has found no adverse effects with HMB-FA (5, 7, 184). Some issues have been raised towards the safety of HMB in rats, due to a possible negative effect on plasma insulin, which was found increased after 320 mg/ kg body weight.day^{-1} for one month (185). This may suggest, in fact, a possible decrease in insulin sensitivity, albeit no

research in humans has confirmed these results. Conversely, another recent research in rats shows protective effects of HMB upon insulin resistance, induced by a high-fructose diet (186). It may be prudent to conduct further research on the effects of HMB upon insulin sensitivity in humans, to clarify this issue.

Another issue that has been raised, is related with the low availability of amino acids, specially glutamine, observed after HMB administration to rats (187). The importance of glutamine for proper gut and immune cell function has been shown, thus low availability to visceral organs might be an issue (155), especially in individuals exhibiting symptoms of critical illness (188, 189). In addition, low levels of glutamine may itself directly impair MPS (189, 190). However, HMB seems to increase MPS (20), thus reduced glutamine levels may be of minor (if any) importance.

Although safety issues have been raised towards reduced insulin sensitivity and lower glutamine levels, research reviews in humans have suggested HMB to be safe both in critically ill patients and healthy individuals (43, 152, 153, 164, 191).

DURATION OF SUPPLEMENTATION, DOSE AND TIMING

It is unfeasible to consume HMB from foods to attain the 3 g daily dose. To put this in perspective, it would require 600 g of high quality protein (to supply ≈60 g of leucine) in order to obtain 3 g of HMB (49). The minimal effective dose reported, with resistance exercise, is 1.5 g/day - with 3 g per day offering some benefits regarding FFM gains - while a 6 g daily dose does not seem more effective (192). This was previously shown by Slater & Jenkins (48) and Wilson et al. (193), again with resistance exercise, when these authors observed that 3 g were superior to 1.5 g daily, in ameliorating both body composition, strength and indirect markers of muscle damage.

To further improve HMB retention, splitting the 3 g daily dose into three doses of 1 gram is recommended (typically at breakfast, lunch and dinner) (174). Most studies using HMB-Ca have in fact used 3 g daily doses (4, 35, 39, 182, 194-217) while some have compared different dosing protocols (42, 218). This led most studies to apply the same supplementation protocol, while using HMB-FA - 3 g daily, typically split in three daily doses of 1 g (5, 7, 20, 184, 219-234). From all the reviewed literature it seems that

3 g would be sufficient to improve body composition, performance, biochemical markers of muscle damage and inflammation (151, 162, 164). Although it has been suggested by Wilson et al. that 3 g of HMB-Ca should be taken, at least 60 min prior to intense exercise (if consumed with glucose - 120 min prior to exercise), HMB-FA should be taken 30-60 min prior to exercise (purportedly due to the different absorption kinetics - figure 6) (151). Altogether, the current body of evidence suggests that the total amount, not the timing, are of primary importance regarding HMB effects.

Insofar as the duration of the supplementation protocol is concerned, this seems dependent on the level of training of each individual. Young untrained subjects and the elderly, typically require only 3-6 weeks to increase 0.5-1 kg greater then controls (35, 42, 48, 49, 174, 181, 192), while trained subjects require a longer time of supplementation, usually 6-12 weeks (5, 7, 210, 212, 215, 217). This seems true for both forms of HMB and intense RE seems mandatory to elicit these results (164).

PROPOSED MECHANISMS OF ACTION

HMB might influence skeletal muscle mass by affecting: a) Protein metabolism (proteolysis inhibition, attenuating depression in protein synthesis, promoting protein synthesis); b) Cholesterol synthesis; c) GH/IGF-1 axis expression; d) Myogenic cell proliferation; e) apoptosis inhibition (figure 7). Most of these mechanisms have been described in animal models and/or *in vitro* research, with few confirmative studies being performed in humans. These mechanisms will be briefly reviewed, with information from human research studies, when available.

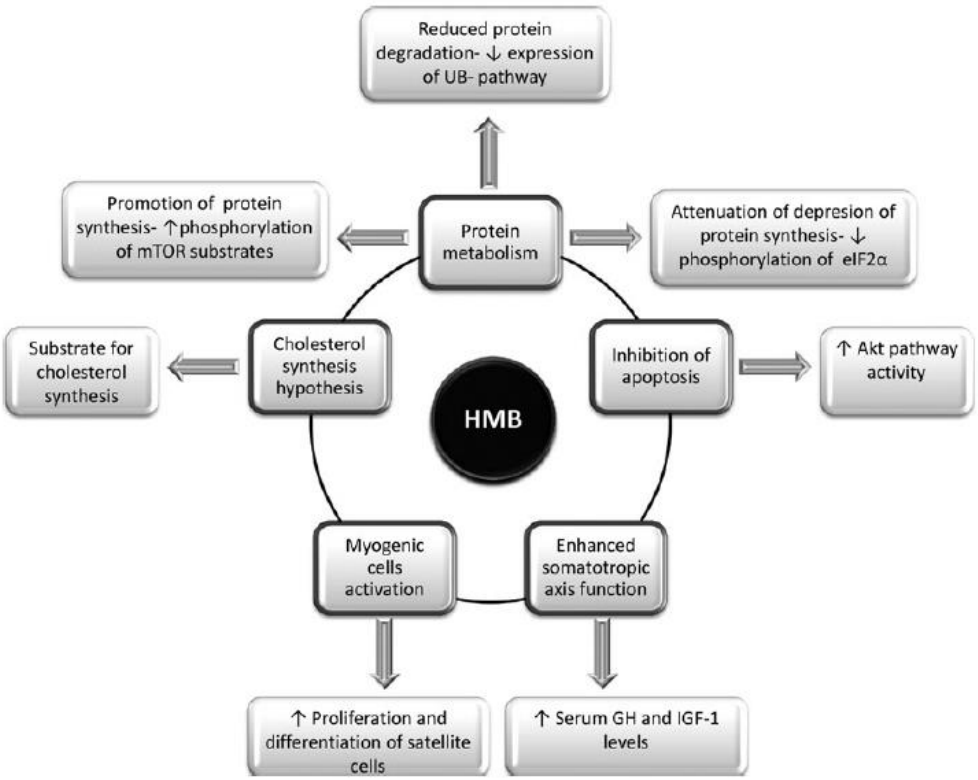

Figure 7. Proposed mechanisms of action for HMB adapted from (166).

Protein metabolism

Some research studies have proposed that HMB might enhance MPS via mTOR (142). This was shown by increased phosphorylation of downstream proteins of the mTOR pool of kinases (p70S6K and 4E-BP1), which were completely attenuated by the mTOR inhibitor rapamycin (142). Albeit other researchers have confirmed this pathway (235, 236), in humans, these mechanisms have never been fully explored. It is thought that HMB might act upon mTOR through common pathways to leucine or growth factors (figure 8). However, not all research showed increased protein synthesis with HMB. Kovarik et al. and Holecek et al. failed to demonstrate any effect on protein synthesis of both muscle and visceral organs of rats (187, 237).

In humans, HMB-FA has been shown to elevate MPS similarly to leucine (20), with no differences between both forms of HMB (228). It is unclear at this point if HMB stimulates mTOR directly, via increased expression of insulin-like growth factor 1 (IGF-

1) or other leucine metabolite (43). In addition to stimulate MPS, HMB might inhibit the depression of MPS. *In vitro* research has shown that when stimulating RNA-dependent protein kinase (PKR) either by administering proteolysis inducing factor (142), TNF-α, angiotensin II or lipopolysaccharides (70, 71), an increased phosphorylation of eukaryotic initiation factor 2 α (eIF2α) occurred, which impaired translational efficiency and protein synthesis. However, when administering HMB, the phosphorylation of eIF2α or the response to PKR was attenuated (70, 71, 142). This led some authors to suggest a possible positive action of HMB on several catabolic conditions and clinical populations (70, 71, 142, 238), with this being further proposed by several reviews and meta-analysis performed in humans (41, 146, 152-157, 159, 191).

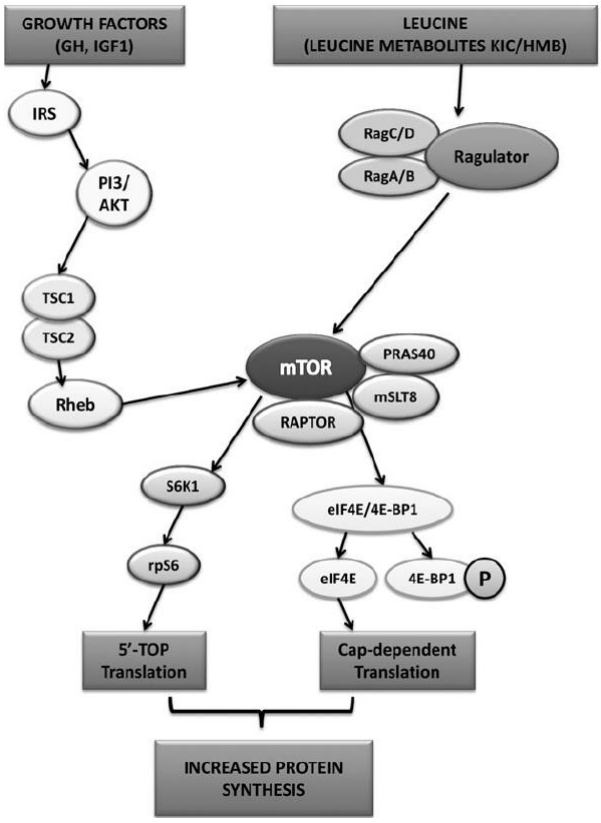

Figure 8. Possible mechanisms regarding HMB and increased protein synthesis adapted from (166)

Both TNF-α and angiotensin II may phosphorylate PKR through caspase-8 and caspase-3 enzymes. After being phosphorylated, PKR will enhance reactive oxygen species (ROS) production via P38 mitogen-activated protein kinases (P38 MAPK) which

will increase the expression of NF-κB and increase protein degradation via the ubiquitin proteasome pathway (UPP). Simultaneously, the auto phosphorylation of PKR may lead to the phosphorylation of eIF2α, which will hinder protein synthesis (reducing translational efficiency) (figure 9). It has been shown that HMB may decrease the activity of caspase-8 and 3, thus decreasing the protein degradation pathway (71). It seems that HMB prevents the depression of protein synthesis as a result of increases in lipopolysaccharides, TNF-α and angiotensin II (70). However, although earlier human research studies and reviews have suggested that HMB might inhibit protein degradation (42, 43, 151), more recent research studies have questioned these findings (5, 7, 234).

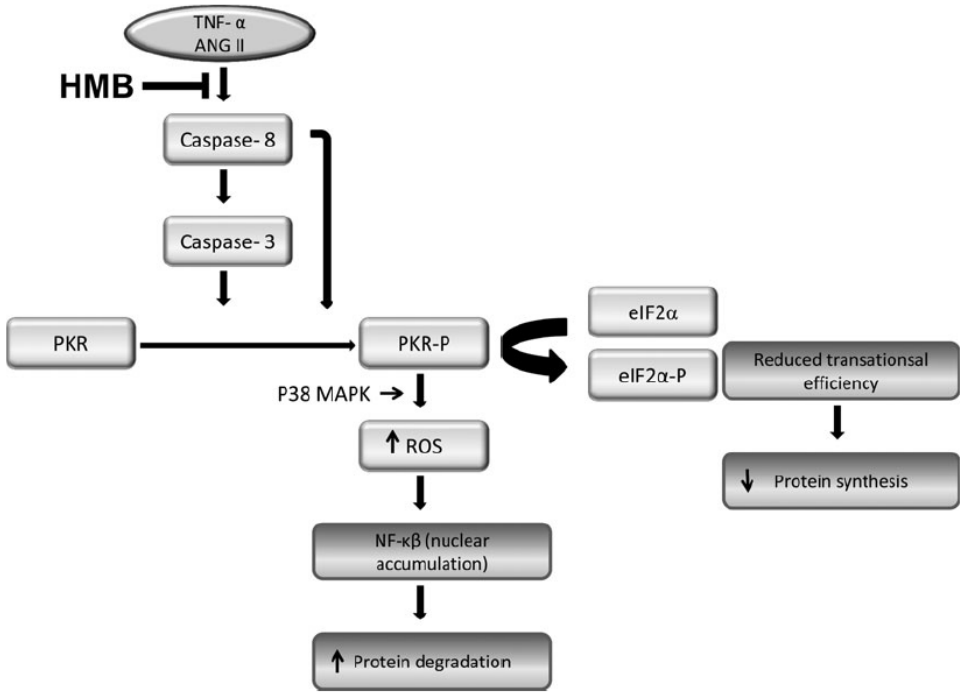

Figure 9. Positive actions of HMB by inhibiting PKR on protein degradation and synthesis adapted from (166)

Additionally, it has also been suggested that HMB might prevent muscle atrophy (235) since *in vitro* treatment of cultured L6 myotubes with HMB supressed the expression of ubiquitin ligases atrogin-1 and MuRF-1, while increasing the phosphorylation of P38 MAPK, PI3K and mTOR (235). Further *in vivo* research studies are commended to clarify if HMB might in fact prevent muscle atrophy. From a

mechanistical stand point (mainly derived from *in vitro* and animal model studies), HMB might affect protein metabolism. However, few studies have been performed in humans to confirm these mechanisms.

Cholesterol synthesis

As previously stated, the cell sarcolemma relies on *de novo* synthesis of cholesterol. It has been postulated that under stressful situations the muscle may lack the capacity to properly synthesise cholesterol (166). This may be of primary importance since cholesterol is essential for proper cell function. Since 1954, HMB has been proposed to be the main precursor of cholesterol synthesis, donating its carbon structure to the cholesterol molecule (239-241). One hypothesis that was raised, is that HMB might offer advantages for proper cholesterol production in the muscle fibre, further stabilizing the sarcolemma (31).

Since in some studies, HMB decreased the post-exercise levels of proxy markers of muscle damage (i.e. CK, lactate dehydrogenase [LDH]), some authors have postulated that this may be the result of enhanced muscle cell membrane function. Both human studies (4, 5, 7, 35, 39, 42, 195, 198, 219, 220, 222, 242) and animal studies as reviewed by Szczesniak et al. (166), seem to suggest that HMB might reduce these proxy markers of muscle damage, although not all research confirms this action in humans (193, 194, 199, 202, 210, 211, 215, 217, 218, 223). The effect of HMB on stabilizing the cell membrane, possibly reducing muscle damage, seems of minor importance, since its effects on protein metabolism (as previously described) are likely more significant, at least in animal research studies (166).

GH/IGF-1 axis expression

HMB has been proposed to influence the GH/IGF-1 axis in humans and animals (43, 166). These two hormones contribute to the somatotrophic axis and may influence metabolism and growth (243). This has been shown in animals receiving HMB during pregnancy (243, 244). Increases in pituitary and hepatic mRNA for GH and IGF-1 have been further noticed in rats (40). Albeit some animal studies have shown increased levels for these hormones, with HMB supplementation, others have failed to observe significant changes in broiler chickens (166, 245).

Although some *in vitro* studies have been performed with HMB and human cells (myoblasts) (246), the current body of evidence for experimental *in vivo* human studies is equivocal, since some studies have detected increases in both hormones with HMB supplementation and resistance exercise training (RET) (4, 247), while others have failed to observe any significant changes (39, 217, 230).

Myogenic cell proliferation

The evidence regarding the possible role of HMB upon myogenic regulatory factors, as previously described by some reviews (43, 151), results from *in vitro* or *in vivo* animal studies (246, 248-251). In fact, Kornasio et al. (246) showed, in chicken myoblast cultures, an elevation of mRNA for myogenic differentiation factor (myoD), which is commonly accepted as a marker of activated satellite cells. In the same study, myosin heavy chain was also increased and more nucleus were incorporated in the myotubes.

Additionally, in rats, these results were confirmed after 14 days of hindlimb suspension, from the analysis of explanted muscle (248). This *in vivo* work, also confirmed an increase in MyoD/Myogenin-positive nuclei in rat muscle. Whether this enhancement in satellite cell mitotic activity is the result of an increase in GH/IGF-1 expression from HMB (252), or the result of other direct unknown mechanism upon satellite cells, remains unclear at this point.

IMPROVEMENTS IN BODY COMPOSITION, STRENGTH, POWER AND BLOOD MARKERS

Untrained subjects

In the seminal work by Nissen et al. (42), the effect of supplementing with several doses of HMB was assessed for three weeks in untrained subjects, while performing a RET protocol. Albeit no results were found regarding body composition, a dose dependent increase of HMB in plasma was noted (+5% with 1.5 g daily, +10.4% with 3 g daily, when comparing with the placebo group). Moreover, HMB also significantly decreased the 3-methylhistidine (3-MH) levels at weeks 1 and 2 of the study, suggesting a lower protein catabolism in the supplemented groups. The authors proposed that this

decrease might have been the result of a reduction in proxy markers of muscle damage (CK, LDH).

Gallagher et al. (253), in a subsequent study, administered different amounts of HMB (38 mg/kg.day^{-1} vs 76 mg.kg.day^{-1}) to untrained subjects for eight weeks, introduced to RET. No differences were found between groups regarding 1 repetition maximum (1RM) strength, albeit the HMB groups increased ≈12% their strength levels when comparing with the placebo group. Interestingly, only the 38 mg/kg.day^{-1} increased FFM (+1.9 kg) while no changes were noted in the higher intake group. Jowko et al. (198) further provided evidence for the benefits of 3 weeks supplementation with 3 g daily, in untrained subjects. However, it should be noted that albeit a trend towards an increase in FFM was noted with HMB, only the combination of HMB+creatine significantly increased FFM. Interestingly, an attenuation in the increase of CK was noted with HMB in this study, in line with the previous work from Nissen et al. (42). A previous study by Panton et al. (35), had already shown that improvements in strength and body composition were more common in untrained individuals when comparing with trained individuals. Still, it should be noted that improvements in body composition were only noted when combining men and women evaluated by underwater weighting ([UWW)] - FM decrease and FFM increase), since no differences were noted using skinfolds (SKF).

Insofar as markers of muscle damage and inflammation are concerned, Wilson et al. (193) failed to find any significant differences, between groups, when supplementing untrained subjects with HMB, after an acute protocol (72 h) of muscle damage. No differences were found for muscle soreness, maximum voluntary contraction, CK or LDH (although HMB might prevent an increase post-exercise). Conversely, in a longer duration study (12 weeks - under a resistance training protocol), Kraemer et al. (208), found a significant decrease, in untrained subjects, regarding interferon gamma (IFN-γ), IL-10 and interleukin 1 beta (IL-1β), with HMB supplementation.

More recently, two studies with HMB-FA, have further added to the current body of literature regarding untrained subjects (230, 233). Albeit with different assessments, both works are seemingly from the same cohort of subjects. Asadi et al. (230), showed that after six weeks of RET and 3 g of HMB daily, higher increases in VJ power output, 1RM leg press and body mass were noted when comparing with the placebo group.

Regarding blood markers, a significant reduction was noted regarding adrenocorticotrophic hormone (ACTH) and cortisol after the RET program (48 h after the last training session), with no differences being observed, between groups, for anabolic hormones (IGF-1, testosterone and GH). Additionally, in the same group of subjects, Arazi et al. found no differences between groups pertaining malondialdehyde, 8-hydroxy2-deoxyguanosine, protein carbonyls, ALT, AST, ALP or white blood cells (233). A possible action of HMB upon markers of muscle damage and inflammation seems unclear at this point, definitely warranting further research.

Recreationally active subjects

Few studies have investigated the effects of HMB on recreationally active subjects. Kraemer et al. (4) presented the most extraordinary results to date, regarding body composition and performance, following 12 weeks of supplementation with HMB and other amino acids (3 g HMB + 14 g arginine + 14 g glutamine daily). These authors reported increases of 40% FFM, 85% muscle power, 40% muscle strength, while simultaneously reporting decreases of 40% FM after these 12 weeks of supplementation under a non-linear periodized RET protocol. To put these findings in perspective, the HMB supplemented group gained ≈9.3 kg of FFM while losing FM simultaneously. Additional findings in this study were related with increases in testosterone and GH, and decreases in cortisol, CK and malondialdehyde.

More recently, Shirato et al. (211) also studied 18 recreationally active male subjects, while supplementing with 36.6 g whey and 3 g HMB-Ca daily. No statistically significant differences were found between groups (Whey + HMB; HMB or Whey groups) regarding variations in isometric strength, muscle soreness or proxy markers of muscle damage (CK, LDH), albeit the HMB group displayed a significant increase in handgrip strength and muscle density. It should however be noted that the Shirato et al. study (211) had a shorter duration (11 days), a different RET protocol (only eccentric exercise) and did not control participants' dietary intake.

Trained and competitive athletes

The first study to display interesting results regarding trained athletes was published by Nissen et al. in 1996 (42). After seven weeks of 3 g of HMB-Ca daily, 32

trained male football players significantly decreased protein catabolism (3-MH) and CK, while increasing weight and FFM. Later, Kreider et al. performed two studies (194, 218) with trained subjects using 3 or 6 g of HMB-Ca daily for 28 days. In both studies, HMB-Ca was not superior to placebo regarding 1RM performance, body composition (218), sprint performance, lifting volume, creatinine, urea N, urea N/creatinine, uric acid, CK, LDH, ALT or AST (194). Additionally, 6 g daily were not superior to 3 g daily, which led to the use of the 3 g daily dose in almost all subsequent studies, with few exceptions (254, 255).

Elite male rugby players and college football players were further investigated when supplementing with 3 g HMB daily for six weeks and four weeks, respectively (201, 203). O'Connor & Crowe (203), in professional rugby league players (minimum four years RET experience), administered either HMB (3 g daily) or HMB+creatine (3 g each daily). Athletes were subject to a full body RET protocol (three days per week), one speed/power session, and four condition/skill session, during six weeks. No difference on any parameter compared with presupplementation measures or the control group were found pertaining muscular strength (3RM bench or deadlift), muscular endurance (pull-ups to failure) or body composition. These results are similar to previous research investigations in competitive college football players (28 days protocol), as previously described (194, 218) with further research by Ransone et al. (201), also failing to find any significant findings insofar as strength (1RM bench press, squats and power cleans) and body composition (body mass and body fat). Since these were trained athletes, it was postulated by some reviews, that periods longer than six weeks would be required to detect significant findings in body composition or performance (151, 162, 164), since trained athletes usually plateau their performance shortly after off-season (256). In fact, the majority of studies performed under six weeks with trained athletes, using both HMB-Ca (199, 200, 202, 242, 257) or HMB-FA (226, 229), did not seem to find significant results.

Longer duration studies (>6 weeks) tended to find significant changes in body composition and performance. This seems confirmed by studies using HMB-Ca in resistance trained athletes (206), combat sport athletes and rowers (210, 215, 258) and also in HMB-FA studies on resistance trained athletes (5, 7, 184). Recently, Durkalec-Michalski et al. (215), showed in competitive combat athletes an increase in FFM and a

decrease in FM with HMB-Ca. This is in line with other research studies, performed in female judo athletes (although no increase in FFM was noted with HMB-Ca, only a decrease in FM) (259).

Wilson et al. (7) reported some of the most extraordinary findings with HMB-FA supplementation to date, in resistance trained athletes. He randomly assigned 20 young resistance trained men, using the same supplementation protocol and training protocol as a previous paper (222) but for a longer period (12 weeks). Several biochemical markers, body composition, and peak power markers were assessed. All subjects underwent 12 weeks of periodized RET. The supplemented group increased more total strength (bench press, squat, and deadlift combined) and vertical jump over the 12 week period, when comparing with the placebo group, as expected from previous research. However, the most controversial results are related to the magnitude of increase in FFM, with 7.4 ± 4.2 kg being reported to the HMB-FA group vs only 2.1 ± 6.1 kg in the placebo group. The supplement also reduced more body fat (-5.4 ± 1.6 kg vs -1.7 ± 2.7 kg in the placebo group). Cortisol and CK increases were also attenuated in the supplemented group during the overreaching phase (weeks 9 and 10), when comparing with the placebo group. HMB-FA also further improved the participants' perceived recovery from the previous bouts of exercise, when comparing with the placebo group. One possible mechanism that might explain this anabolic effect reported by Wilson et al. (7) with HMB-FA is the increase in GH levels, when combined with high volume resistance training, as reported by Townsend et al. (247), however this hormone was not assessed in this study.

More recently, Lowery et al. (5) also reported extraordinary results in regards to increases of FFM. This protocol used HMB-FA in the same fashion as the previous work by Wilson et al. (7) but included 400 mg of ATP per 1 g of HMB-FA. In the HMB-FA+ATP group, muscle power was enhanced as per the results elicited by the Wingate and vertical jump tests (23.7 % and 21.5 % respectively). During the overreaching phase of the periodized RET protocol, strength and power declined in the placebo group (4.3-5.7 %), whereas supplementation with HMB-FA+ATP resulted in continued strength gains (1.3 %). After 12 weeks of this RET protocol, HMB-FA+ATP increased FFM by 8.5 ± 0.8 kg vs 2.1 ± 0.5 kg in the placebo group and reduced FM by 8.5% vs 2.4% in the placebo group. From this study, the authors also concluded that HMB-FA can alone

decrease CK after the overreaching cycle, albeit not showing significant effects in urinary 3-MH levels. Improved perceived recovery was also reported in the HMB-FA+ATP group throughout the study (at weeks 1, 4, 9, 10, 12).

The increases in FFM over 12 weeks, reported by Lowery et al. (5) and the Wilson et al. (7) studies, even with periodized RET, are virtually unprecedented in the literature with just HMB or any other supplement, with the possible exception of results reported by Kraemer et al. in recreationally active individuals (208) by combining HMB-Ca with other supplements like glutamine, arginine and taurine. Recent letters to the editor have questioned some methodological aspects of these research studies, encouraging their replication due to inconsistencies from the data previously published from other laboratories (260-262).

As previously mentioned, studies in trained athletes require longer duration protocols (typically > 6 weeks) (43, 151, 164). However, even with these experimental conditions some studies have failed to detect significant differences between HMB and placebo groups. This has been reported in both resistance trained athletes over nine weeks (205) and in elite rugby players over 11 weeks (214). Thomson et al. (205), failed to detect any body composition improvements (using bioelectrical impedance [BIA] and SKF) in resistance trained men, after nine weeks of a RET protocol (only lower body increases in strength were detected). Similar results were reported by McIntosh et al. (214) in elite rugby players, during pre-season preparation, with no differences regarding body composition (except for a small significant increase in body mass) or strength (bench press, cleans, squat and weighted pull ups) being detected. Surprisingly, the HMB supplemented group displayed hindered performance on the Yo-Yo performance test.

A recent study by Jakubowski et al. (217), compared HMB to leucine with the addition of 25 g whey protein, over 12 weeks. No differences were found between HMB and leucine, pertaining body composition (dual-energy X-ray absorptiometry [DXA], ultrasound [US], muscle cross-sectional area [CSA], total body water [TBW]), 1RM, Wingate power, markers of muscle damage (CK, LDH) or hormones (cortisol, IGF-1, GH, testosterone). Importantly, this well designed study, used the same RET protocol as previously used by Wilson et al. (7), Lowery et al (5) and Kraemer et al. (4), with each group gaining only 2.3 and 2.6 kg of lean soft tissue (LST), respectively.

Due to the extraordinary results reported with both HMB-Ca (4) and HMB-FA (5, 7), regarding body composition and performance, and also due to the equivocal results regarding proxy markers of muscle damage and inflammation (4, 5, 7, 42, 193, 194, 199, 202, 206, 208, 211, 215, 217-223, 226, 229, 230, 234, 242, 247, 258), direct comparative studies between both forms of HMB, in resistance trained individuals, are commendable.

Currently meta-analysis and systematic reviews, also display mixed results insofar as HMB supplementation is concerned. Some have pointed towards positive results (performance and body composition enhancement, attenuation of markers of muscle damage) in untrained and trained individuals (143, 148, 150) while others show no benefit (144, 149). This contrasts with the optimism of most narrative reviews (43, 155, 163, 164, 222), albeit some have labeled as implausible the drug-like actions of HMB-FA (160). Also, most of these meta-analysis have been strongly driven by the previously mentioned research studies with extraordinary results (4, 5, 7).

Recently, one meta-analysis suggested that HMB could significantly reduce proxy markers of muscle damage (CK an LDH) and that periods superior to six weeks are required to reduce exercise-induced muscle damage (EIMD) (150). This meta-analysis is in agreement with a previous systematic review by Silva et al. (148). Another narrative review has suggested HMB as containing anti-inflammatory and antioxidant actions (233). Again, in our view, and as previously mentioned, these results require further confirmatory research.

For clarity, table 1 and table 2 encompasses studies performed in humans, using HMB-Ca and HMB-FA, in young healthy individuals.

Table 1. Experimental studies with HMB-Ca in young healthy individuals

Reference	Subjects	Duration/ Dose	Exercise Protocol	Assessments	Main Findings vs Placebo or other group
Nissen et al. 1996 (42)	32 Trained male football players	7 wks, 3 g HMB daily	Monitored progressive RET	TOBEC, bench press, squat, urine, blood	↓ 3-MH, CK ↑ Weight load (study1) ↑ FFM ↓ FM
Nissen et al. 1996 (42)	41 Untrained college-aged males	3 wks, 1.5 or 3 g HMB daily	Monitored progressive RT	TOBEC, average weight lifted	↑ Strength ↑ FFM = FM

Reference	Subjects	Duration/ Dose	Exercise Protocol	Assessments	Main Findings vs Placebo or other group
Kreider et al. 1999 (218)	40 experienced resistance-trained males	28 d, 3 or 6 g HMB daily	RET, not controlled	DXA, blood, urine, bench press and leg press	= Creatinine, urea N, urea N/creatinine, uric acid, CK, LDH, ALT, AST = 1RM = FBFM, FM, FM%
Kreider et al. 2000 (194)	28 NCAA football players	28 d, 3 g HMB daily	Resistance and agility training	DXA, BIA, blood, sprint performance, bench press, squat, power cleans	= Creatinine, urea N, urea N/creatinine, uric acid, CK, LDH, ALT, AST = Body composition = Sprint performance = Lifting volume
Gallagher et al. 2000 (192)	37 untrained, college-aged men	8 wks, 3 g or 6 g daily	Supervised RET	Isometric strength, SKF, blood, RM	= 1RM ↑ Peak Isometric strength = fatigue evaluation ↓ CK (first 48 h) ↑ FFM (3 g daily not 6 g daily)
Knitter et al. 2000 (195)	16 trained subjects (males and females)	6 wks, 3 g HMB daily	Running 48h/wk	Blood collection	↓ CK, LDH = FFM, FM
Panton et al. 2000 (35)	84 (43 men, 41 women) trained and untrained	4 wks, 3 g HMB daily	Supervised RET	bench press (both genders), leg press (men), leg extension (women)	**Men:** = Strength gains ↑ Upper body strength **Women:** =Strength gains ↑ Upper body strength **Combined:** ↑ Upper body strength
Vukovich et al. 2001 (196)	8 cyclists	6 wks, 3 g HMB daily	Cycling training	Graded exercise test, UWW	= VO_2peak, lactate ↑ Time to reach VO_{2peak} ↑ OBLA
Jówko et al. 2001 (198)	40 males untrained	3 wks, 3 g HMB daily vs Cr	Progressive RET	SKF, DXA, CT	↓ CK, Urine urea nitrogen, Plasma urea ↑ FFM when combined with Cr, HMB alone only displays a trend towards an increase of FFM
Slater et al. 2001 (199)	17 (water polo squad) + 10 (national level male rowers)	6 wks, 3 g HMB daily	Regular training routine	3 RM (bench press, leg press), chin-ups, blood, urine, DXA	= FFM, FM, BM, = Strength = CK, LDH, testosterone, cortisol, serum urea, creatinine, urea N/creatinine, 3-MH

Reference	Subjects	Duration/ Dose	Exercise Protocol	Assessments	Main Findings vs Placebo or other group
O'Connor & Crowe 2003 (200)	27 Elite male rugby players	6 wks, 3 g HMB daily	Progressive RET	Multistage test to assess aerobic power, 60 s maximal cycle for anaerobic capacity	= aerobic and anaerobic capacity = Peak lactate
Ransone et al. 2003 (201)	35 College football players	4 wks, 3g HMB daily	Progressive RET and endurance exercise	1RM (bench press, power cleans, squat), 12 x 6 s sprint	= Bench press, squats, and power cleans = Body composition (BM, FM)
Hoffman et al. 2004 (202)	26 male football players	10 days, 3 g HMB daily	Regular training routine	Subjective perception questionnaire, blood, Wingate test	= Anaerobic power = Subjective perception (soreness) = Testosterone, cortisol, CK, myoglobin
O'Connor & Crowe 2007 (203)	30 Trained rugby players	6 wks, 3 g HMB daily	Progressive RET	SKF, 1RM (squat, bench press and deadlift), Wingate	= Muscular strength = Endurance = Body composition
Lamboley et al. 2007 (204)	16 active college students	5 wks, 3 g HMB daily	Interval training on a treadmill 3 x week	Incremental test to exhaustion, DXA	↑ VO_{2max} ↑ RCP = Body composition
Kraemer et al. 2009 (4)	17 recreationally active, college-aged males	12 wks, 3 g daily HMB	Non-linear Periodized RET	DXA, limb circumference, 1RM (squat and bench press), vertical jump, blood	↑ Muscle power (85% increase) ↑ Muscle strength (≈40 increase) ↑ FFM ↓ FM ↑ Testosterone, GH ↓ Cortisol, CK, malondialdehyde
Thomson et al. 2009 (205)	22 resistance trained men	9 wks, 3 g HMB daily	RET protocol	1 RM, BIA, SKF	↑ Lower body strength = Body composition
Wilson et al. 2009 (193)	16 untrained men	72 h, 3 g HMB before and after exercise	2 x 55 maximal eccentric knee flexion and extension	Blood, MVC	= Muscle soreness = MVC = CK, LDH (might prevent post-exercise increase)
Portal et al. 2011 (206)	29 adolescent volleyball players	7 wks, 3 g HMB daily	Regular training routine	SKF, 6 RM, isokinetic force, Wingate, 20 min shuttle run test	↑ FFM ↑ Isokinetic force ↑ Peak and mean power = fatigue index = aerobic capacity = GH, IGF-1, cortisol, IL-6, IL-1
Kraemer et al. 2014 (208)	17 healthy, college-aged men	12 wks, 3 g HMB daily	Non-linear periodized RET	Blood	↓ IFN-γ ↓ IL-10 ↓ IL-1β
Kraemer et al. 2015 (242)	13 resistance trained young men	3 wks, 3 g HMB daly	Heavy RET + metabolic RET	Blood, vertical jump	↑ Vertical jump power ↓ Muscle soreness ↓ CK, IL-6

Reference	Subjects	Duration/ Dose	Exercise Protocol	Assessments	Main Findings vs Placebo or other group
Durkalec-Michalski & Jeszka 2015 (210)	16 elite male rowers	12 wks, 3 g HMB daily	Rowing preparation	Blood, progressive test for $VO_{2\ max}$ assessment, Wingate	↑ VO_{2max} ↓ FM ↑ Time to attain VT ↑ Progressive test (maximum load) = CK, LDH, testosterone, cortisol, T/C
Shirato et al. 2016 (211)	18 recreationally active male subjects	11 days, 3 g HMB daily	Eccentric exercise	Isometric muscle strength, VAS, blood	= Isometric strength = Muscle soreness ↑ Handgrip strength and endurance ↑ Muscle density
Michalski & Jeszka 2016 (258)	58 young highly trained martial arts and rowing athletes	12 wks, 3 g HMB daily	Training routine of each sport	Progressive test for $VO_{2\ max}$ assessment, BIA, blood	↑ Time to attain VT ↑ FFM ↓ FM ↑ Testosterone, cortisol
McIntosh et al. 2016 (214)	27 elite rugby players	11 wks, 3 g HMB daily	Regular training routine	Strength assessments, SKF, Yo-Yo intermittent recovery test	↑ BM = Body composition =Bench press, cleans, squat, weighted pull ups ↓ Yo-Yo test
Michalski & Jeszka 2017 (215)	42 trained combat sports males	12 wks, 3 g HMB daily	Training routine of each sport	Progressive test for $VO_{2\ max}$ assessment, Wingate, blood	↑ Time to attain VT ↑ Anaerobic power ↑ Average power ↑ FFM ↓ FM = CK, LDH, testosterone, lactate
Abad-Colil et al. 2017 (257)	16 male soccer players	4 wks, 3 g HMB daily	Intensified period of in-season soccer training	Anthropometric measures, Squat jump, CMJ, drop jump, Yo-Yo test	= CMJ, drop jump, squat jump = Yo-Yo test = Anthropometric measures
Jakubowski et al. 2018 (217)	23 resistance trained men	12 wks, 3 g HMB daily vs 3 g leucine	Periodized RET	1RM, skeletal muscle biopsies (CSA), DXA, US, blood	= 1RM = Wingate power = TBW, MT, FM. FBFM = CSA = CK, cortisol, IGF-1, GH, testosterone

Abbreviations: **1RM**: 1 repetition maximum; **3-MH**: 3-methylhistidine; **ALT**: alanine aminotransferase; **AST**: aspartate aminotransferase; **BIA**: bioelectrical impedance analysis; **BM**: body mass; **CK**: creatine kinase; **CMJ**: countermovement jump; **Cr**: creatine; **CSA**: cross-sectional area; **CT**: computed tomography; **DXA**: dual-energy x-ray absorptiometry; **FBFM**: fat-bone free mass **FFM**: fat-free mass; **FM**: fat mass; **GH**: growth hormone; **HMB**: β-hydroxy-β-methylbutyrate; **IFN-γ**: interferon gamma; **IGF-1**: insulin-like growth factor 1; **IL**: interleukin; **LDH**: lactate dehydrogenase; **MT**: muscle thickness; **MVC**: maximum voluntary contraction; **OBLA**; onset of blood lactate accumulation; **RCP**: respiratory compensation point; **RET**: resistance exercise training; **SKF**: skinfolds; **TBW**: total body water; **T/C**: testosterone to cortisol ratio; **TOBEC**: total body electrical conductivity; **US**: ultrasound **UWW**: underwater weighting; **VT**: ventilatory threshold; **wks**: weeks

Table 2. Experimental studies with HMB-FA in young healthy individuals

Reference	Subjects	Duration/ Dose	Exercise Protocol	Assessments	Main Findings vs Placebo or other group
Dunsmore et al. 2012 (184)	20 young trained men	12 wks, 3 g HMB daily	Full body periodized RET	Total strength (squat, bench press, deadlift), Wingate, US, urine, blood	↑ Total strength ↑ Peak power ↑ MT
Sikorski et al. 2012 (219)	20 young trained men	48 h, 3 g HMB daily	High volume RET	Blood, PRS	↓ CK ↓ Perceived exertion = Testosterone, cortisol
Davis et al. 2012 (220)	20 young trained men	2 wks, 3 g HMB daily	High volume RET	Blood, Wingate, 1RM (bench press, squat and deadlift)	↓ CK = Strength and power ↓ Cortisol (wks 1 and wks 2) = Cortisol
Townsend et al. 2013 (221)	40 young trained men	48 h (4 sessions), 3 g HMB daily	Acute RET	Blood	Attenuated TNF-α (after training) Attenuated TNFR-1 (recovery period)
Wilkinson et al. 2013 (20)	15 young healthy men	5 h, 3.42 g HMB or leucine	None	Blood, muscle biopsies	↓ MPS vs leucine ↓ mTOR pool of kinases ↓ MPB (independently of insulin)
Wilson et al. 2013 (222)	20 resistance trained men	48 h, 3 g HMB daily	High volume RET	Blood, urine, PRS	↓ CK ↓ Perceived exertion = 3-MH = Testosterone, fT, Cortisol, CRP
Wilson et al. 2014 (7)	20 trained men	12 wks, 3 g HMB daily	Non-linear-periodized RET	DXA, blood, urine, US, 1RM, Wingate, VJ, PRS	↑ Total strength, VJ ↑ Wingate Peak Power ↑ FFM, Quad MT ↓ FM ↓ CK, cortisol ↓ Perceived exertion
Gonzalez et al. 2014 (224)	39 young trained men	48 h, 3 g HMB daily + CWI	RET	Blood, Maximal strength test	30 min after: HMB = CWI 48 h after: ↑ CR3 in monocytes = recovery and performance
Gonzalez et al. 2014 (223)	40 young trained men	48 h, 3 g HMB daily + CWI	RET	PRS, performance measures (strength)	= CK ↓ CRP ↑ Performance (only HMB + CWI)
Robinson et al. 2014 (225)	34 young untrained individuals	4 wks, 3 g HMB daily	HIIT	Incremental test to volitional exhaustion, DXA	↑ VO_{2peak} ↑ VT = RCP, Time to exhaustion = FBFM, FM

Reference	Subjects	Duration/ Dose	Exercise Protocol	Assessments	Main Findings vs Placebo or other group
Townsend et al. 2015 (247)	20 young trained men	30 min, 1 g HMB	Progressive RET	Blood	↑ GH, IGF-1 = Testosterone, insulin
Lowery et al. 2016 (5)	17 trained young men	12 wks, 3 g HMB daily + 400 ATP	Non-linear-periodized RET	DXA, blood, US, 1RM, Wingate, VJ, PRS	↑ Total strength ↑ Vertical jump ↑ Wingate Peak power ↑ FFM, Quad MT ↓ FM ↓ CK, cortisol =3-MH, fT, testosterone ↓ Perceived exertion
Hoffman et al. 2016 (226)	13 military commandos	23 days, 3 g HMB daily	Intense military training	Blood, MRI	= Muscle mass ↓ TNF-α
Miramonti et al. 2016	37 healthy untrained individuals	4 wks, 3 g HMB daily	HIIT	PWCFT	↑ PWCFT
Wilkinson et al. 2017 (228)	8 healthy young males	5 h, 3.42 g HMB-Ca vs HMB-FA	None	Blood, muscle biopsies	≠ bioavailability = MPS, MPB, mTOR stimulus
Redd et al. 2017 (229)	13 military commandos	23 days, 3 g HMB daily	Intense military training	Blood	= IGF-1, IGFBP-1, IGFBP-2, IGF-BP3, IGF-BP4, IGF-BP5, IGF-BP6 ↓ IGF-BP7
Asadi et al. 2017 (230)	16 healthy, untrained men	6 wks, 3 g HMB daily	RET	1 RM (bench press, leg press), VJ, blood, anthropometric measures	↑ VJ power ↑ 1RM leg press ↓ ACTH, cortisol = GH, IGF-1, Testosterone
Correia et al. 2018 (231)	23 trained young males	72 h, one single dose of 3 g HMB	Muscle damaged protocol	US, CMJ, MVIT, WC	↑ WC = Muscle swelling = MVIT = CMJ
Arazi et al. 2018 (233)	16 healthy, untrained men	6 wks, 3 g HMB dailyy	RET	Anthropometric measures, blood	= Malondialdehyde, 8-hydroxy-2-deoxyguanosine, protein carbonyl, ALT, AST, ALP, WBC population
Tinsley et al. 2018 (234)	11 individuals	3 days + fasting, 3 g HMB daily	None	Urine, saliva and blood	=3-MH/CR (= MPB) ↓ Cortisol (30-45 min post-awakening) ↓ T:C

Abbreviations: **1RM**: 1 repetition maximum; **3-MH**: 3-methylhistidine; **ACTH**: adrenocorticotropic hormone; **ALP**: alkaline phosphatase; **ALT**: alanine aminotransferase; **AST**: aspartate aminotransferase; **CK**: creatine kinase; **CMJ**: countermovement jump; **CR**: creatinine; **CR3**: complement receptor 3; **CRP**: C-reactive protein; **CWI**: Cold water immersion; **DXA**: dual-energy x-ray absorptiometry; **FBFM**: fat-bone free mass; **FFM**: fat-free mass; **FM**: fat mass; **fT**: free testosterone; **GH**: growth hormone; **HMB**: β-hydroxy-β-methylbutyrate; **HMB-Ca**: β-hydroxy-β-methylbutyrate calcium salt; **HMB-FA**: β-hydroxy-β-methylbutyrate free acid; **IGF**: insulin-like growth factor; **IGFBP**: insulin-like growth factor binding protein; **MPB**: muscle protein breakdown; **MPS**: muscle protein synthesis; **MRI**: magnetic resonance imaging; **MT**: muscle thickness; **mTOR**: mammalian target of rapamycin; **MVIT**: maximal voluntary

isometric torque; **PRS**: perceived recovery status scale; **PWCFT**: physical working capacity at the onset of neuromuscular fatigue; **RCP**: respiratory compensation point; **RET**: resistance exercise training; **T/C**: testosterone to cortisol ratio; **TNF-α**: tumor necrosis factor alpha; **TNFR-1**: tumor necrosis factor receptor 1; **US**: ultrasound; **VJ**: vertical jump; **VT**: ventilatory threshold; **WBC**: white blood cells; **WC**: work capacity; **wks**: weeks

OLDER AND DISEASED POPULATIONS

Some studies have shown, that an acute intake (≈3 g) of either form of HMB are able to increase MPS and reduce MPB, to a similar extent than leucine (20, 228). Furthermore, HMB was able to reduce MPB independently of insulin (20, 228). While decreasing MPB has not been recommended in healthy individuals, since the process is essential to promote regeneration of contractile proteins (turnover of 1-2% daily) (263, 264), it has been suggested that excessive protein breakdown, in diseased populations may lead to muscle loss (157, 265, 266). In this regard, a process named anabolic resistance seems responsible, to a certain extent, for muscle loss in elderly populations (267). Interestingly, not only age increases anabolic resistance but also obesity and inactivity (268). This is in agreement with recent findings from Moro et al. (269), where no anabolic resistance was detected in healthy highly active older adults.

Importantly, HMB-Ca, when added to a high protein nutritional supplement has a greater area under the blood concentration-time curve (i.e., net exposure; figure 10) than leucine. It has been proposed that HMB-Ca would have a more sustained effect in stimulating whole-body protein synthesis (WBPS) and decreasing whole-body protein breakdown (WBPB). Furthermore, it has been recently shown that different absorption kinetics exist in older versus younger adults insofar as amino acids and HMB are concerned (270). It should be noted, however, that WBPS is not the same as MPS as the latter accounts for only ≈25% of WBPS (271, 272). Currently, there are no direct studies comparing leucine with HMB, which means the question remains unanswered.

Deutz et al. (207), showed that when HMB was administered for 10 days in bed rest older adults, an attenuation in FFM loss was observed, albeit no differences were found regarding functional parameters. Conversely, when a mixture of EAAs (high in leucine) was administered in a similar design study, no effects on FFM were observed, only functional outcomes (273). However, in another bed rest study, with middle-aged adults by English et al. (274), leucine was protective of whole-body lean mass losses, after seven days of supplementation. From the current body of evidence, regarding bed

rest studies, it is plausible that both compounds may inhibit FFM losses. If any advantages do exist with HMB over leucine, they are likely only detectable in longer term studies, due to the apparent longer half-life of HMB.

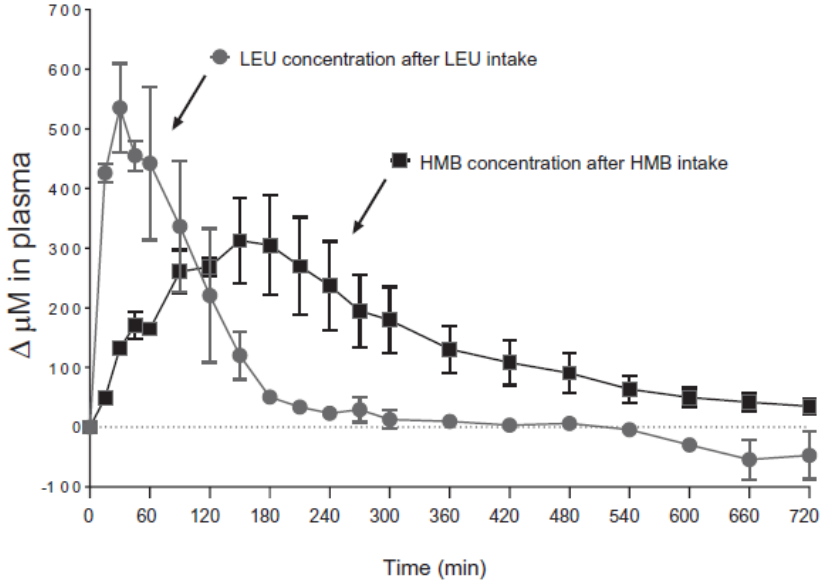

Figure 10. Changes in plasma concentrations after ingesting a high protein supplement and either 3 g of leucine of 1.5 g of HMB-Ca (157)

One of the first human studies with HMB in older adults, was performed by Vukovich et al. (197), in 31 men and women (70 years old), over eight weeks of either HMB or placebo, while performing a progressive RET protocol. Albeit not statistically significant, the HMB supplemented group showed a trend towards an increase in FFM, while a significant decrease in FM percentage was detected. In a subsequent study in 50 elderly women (254), a mixture of HMB (2 g daily)+arginine+lysine over 12 weeks without any training regimen displayed interesting results. Improvements were detected on functionality tests, limb circumference, leg strength, handgrip strength and WBPS. Prolonged HMB-Ca also improved strength, body composition, functionality and muscle quality, with and without exercise (182). Longer duration studies also found positive results, when supplementing HMB-Ca with other ingredients, in preventing mild to moderate sarcopenia in older men and women (275).

When looking into the whole body of evidence (table 3), it seems that no study with exercise rehabilitation or RET displays FFM losses after 10 days and that after eight weeks, an increase in FFM is usually detected. At eight weeks, positive results are already found, even in healthy older women after a mild fitness program, with improvements in some strength assessments but not FFM (255). In diseased populations benefits range from 50% lower mortality in older malnourished hospitalized patients (213), improved muscle strength, FFM and functionality in bronchiectasis patients (especially when combined with pulmonary rehabilitation) (276) to amelioration in postoperatively hip fractures in older women (277) and preserved quadriceps muscle strength after knee osteoarthritis surgery (278). It should also be noted, that not all studies with HMB in diseased populations show positive results (279) and that some limitations have been pointed out to some research (213, 280, 281).

When looking into several meta-analysis and systematic reviews, it seems clear that the evidence is equivocal. While some have found that HMB contributes to the preservation of muscle mass in older adults (145), others have reported inconsistent results for muscle strength and physical performance, with small increments being detected in leg lean mass but not total lean body mass (147). Only one systematic review assessed HMB in clinical settings, suggesting a combination with arginine and glutamine, while reporting that more studies are required to draw more accurate conclusions. Again this is in stark contrast with several narrative reviews pointing out positive results with HMB in both diseased and older populations (152, 155-159, 161, 191). More research studies in diseased populations (i.e. diabetics) are commendable, to further clarify whether HMB or other leucine metabolite might be effective to improve body composition, functionality or reduce mortality in these populations. Also, studies comparing leucine with HMB or other leucine metabolites are warranted.

Table 3. Experimental studies with HMB-Ca and HMB-FA in older and diseased populations

Reference	Subjects	Duration/ Dose	Exercise or rehabilitation Protocol	Assessments	Primary result
Vukovich et al. 2001	31 older man and women	8 wks, 3 g HMB-Ca daily	RET	SKF, DXA, CT	= FFM (tendency to ↑) ↓ % FM

Reference	Subjects	Duration/ Dose	Exercise or rehabilitation Protocol	Assessments	Primary result
Flakoll et al. 2004	50 elderly women	12 wks, 2 g HMB-Ca daily	None	"get-up-and-go" test, BIA, blood and urine	↑ Functionality test ↑ Limb circumference ↑ Leg strength ↑ Handgrip strength ↑ WBPS
Stout et al. 2013	54 older men and women	24 wks, 3 g HMB-Ca	With and without RET	Isokinetic leg extension, handgrip strength, "get-up-and-go" test, DXA, muscle quality	↑ Isokinetic leg extension (RET group) ↑ Muscle quality leg (RET group) ↑ FFM and leg FFM (RET group) ↓ FM (RET group - week 24)
Stout et al. 2015	48 older man	12 wks, 3 g HMB-Ca + 8 g CHO	RET	DXA	↓ Abdominal fat mass (RET group)
Berton et al. 2015	80 healthy older women mildly active	8 wks, 1.5 g HMB-Ca day	Mild fitness program	SPPB, isometric and isokinetic strength, 6-min walk test, handgrip, DXA, CT,	=SPPB ↑handgrip strength =DXA ↑Isokinetic flexion and extension
Nishizaki et al. 2015	23 patients undergoing arthroplasty	5 days before and 28 days after surgery	RET, ROM, Walking	Strength assessment	No loss of muscle strength
Deutz et al. 2016	652 older inpatient, malnourished individuals with several comorbidities	Death or nonelective readmission (90 days) and/or 30-60 day readmission or death, 3 g HMB-Ca daily	None	90-day post discharge incidence, death or nonelective readmission, LOS, BM, (ADL)	= 90 post-discharge ↓ 90 mortality = LOS = ADL ↑ BM
Ekinci et al. 2016	75 older women with hip fracture	30 days, 3g HMB-Ca daily with Vit D_3 and protein	None	Anthropometry, blood, wound-healing, immobilization period, muscle strength	↓ Wound period ↑ muscle strength ↑ ambulatory and mobilization
Cramer et al. 2016	330 men and women malnourished and sarcopenic	24 wks, whey, Vit D_3, 1.5 g HMB-Ca	None	Isokinetic force, strength	↑ leg muscle strength and quality in mild-moderate but not severe sarcopenia
Olveira et al. 2016	30 patients with bronchiectasis	12 wks, 1.5 g HMB-Ca + protein + prebiotic fiber	Pulmonary reabilitation	DXA, arm circumference, BIA, quality of life, handgrip strength, blood	↑ Bone density, handgrip strength, arm circumference, physical functioning, quality of life

Reference	Subjects	Duration/ Dose	Exercise or rehabilitation Protocol	Assessments	Primary result
Fitschen et al. 2017	33 hemodialysis patients	6 months, 3 g HMB-Ca	None	Body composition, bone density, strength, Physical function, fall risk, quality of life, blood	No differences in any parameter
Din et al. 2018	16 healthy older men	6 wks, 3 g HMB-FA day	Unilateral progressive RET	D_2O, muscle biopsies, DXA, US, ID, 1RM	= 1RM = ID = VL thickness ↑ Thigh lean mass (DXA) = MPS = Gene expression
Ellis et al. 2018	31 older community-dwelling man and women	6 months, 3 g HMB-Ca daily	Non-linear-periodized RET	4C (DXA, ADP), MRI, physical function tests	↑ Time stair climb ↑ FFM (DXA, ADP) = MRI (quadriceps)

Abbreviations: 1RM: 1 repetition maximum; **4C**: 4-compartment model; **ADL**: activities of daily living; **ADP**: air displacement plethysmography; **BIA**: bioelectrical impedance analysis; **BM**: body mass **CHO**: carbohydrate; **CT:** computed tomography; **D_2O**: deuterium oxide; **DXA**: dual-energy x-ray absorptiometry; **FFM**: fat-free mass; **FM**: fat mass; **HMB-Ca**: β-hydroxy-β-methylbutyrate calcium salt; **HMB-FA**: β-hydroxy-β-methylbutyrate free acid; **ID**: isokinetic dynamometry; **LOS**: length of stay; **MPS**: muscle protein synthesis **MRI**: magnetic resonance imaging **RET**: resistance exercise training; **ROM**: range of motion; **SKF**: skinfolds; **SPPB**: short physical performance battery; **US**: ultrasound; **VL**: vastus lateralis; **WBPS**: whole-body protein synthesis; **wks**: weeks

2.2.2 Leucic acid (α-HICA)

OVERVIEW

α-HICA, is the hydrogenated metabolite of α-KIC. This by-product of leucine catabolism was initially patented by The John Hopkins University in 1979 (282), being more recently re-patented by Finnish researchers to be used as a food supplement (283). This metabolite has been found in both muscle and connective tissue, within small amounts, not bound to proteins (284, 285). α-HICA has also been described as the result of leucine fermentation in certain foods like cheese, wine and soy (6, 286, 287). After leucine is converted to α-KIC this may be further converted to α-HICA by hydrogenation (figure 11) (287). It has been suggested that the aminotransferase enzyme that oxidizes leucine to α-KIC, might also convert leucine to α-HICA, albeit conditions that determine

the fate of leucine are not clear to date, they seem to be influenced by the oxidoreduction equilibrium (288, 289).

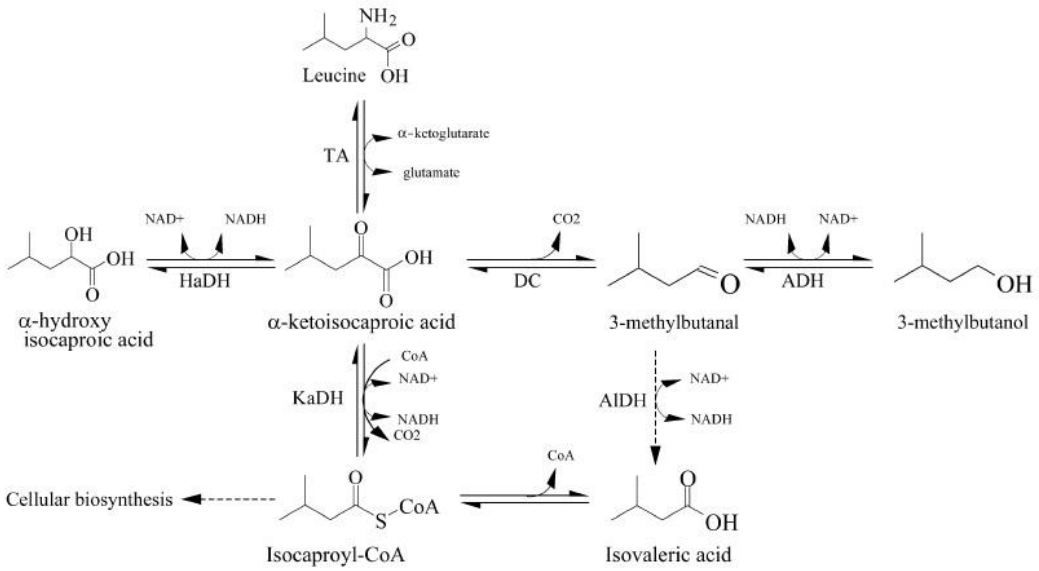

Figure 11. Simplified catabolism of leucine during dairy fermentation by various microorganisms (287)

It has been proposed, from *in vitro* studies, that this compound might present anticatabolic activity, via inhibition of metalloproteinases that may enhance protein degradation in several tissues (6). Moreover, both *in vitro* and animal studies suggest that α-HICA might be anticatabolic (290, 291). Few research has been conducted in humans, some exceptions are the work from Mero et al. (6) and some data mentioned in the patent file, which was not subject to peer review.

HUMAN RESEARCH STUDIES

There is a paucity of studies in humans with α-HICA. Earlier data from the patent file, assessed the effects of ≈500 mg thrice daily, in wrestlers (n=7) after intensive training sessions for six weeks (283). These were top level athletes with at least 10 training sessions per week of 1.5 to 2.5 h. A small increase in mean BM was noted (0.84 ± 1.0 kg) without changes in bone mineral content (BMC), however LST increased significantly. Also, it was importantly noted that delayed onset muscle soreness (DOMS) was reduced when using α-HICA. No changes were observed pertaining blood pressure, heart rate or laboratory blood values, which suggests α-HICA is safe. Major limitations of these data

are related with the absence of peer review and to the small sample used (only seven athletes).

Bearing this in mind, the same group of researchers designed another study, with 15 male soccer players, for four weeks in a double-blinded fashion. The α-HICA group (n=8) received 500 mg α-HICA mixed with liquid thrice daily, while the placebo group (n=7) received 650 mg maltodextrin also thrice daily in the same schedule. No differences between groups were found regarding nutrition, training, blood markers or performance. Insofar as body composition is concerned, a significant increase in BM and FFM was observed in the α-HICA group, with the latter being driven by an increase of 0.4 kg in the lower extremities, while the placebo group decreased FFM in the same body region by 0.1 kg. It was hypothesized, that this increase in the lower extremities was due to the sport (soccer) the subjects were engaged. Importantly, α-HICA supplementation also decreased significantly the whole body DOMS after the 4th week of supplementation, when compared to placebo.

Albeit promising, the data on α-HICA is scant. More research should be performed to assess its alleged effects on preserving FFM, especially in resistance trained men, older and/or diseased populations.

OTHER LEUCINE METABOLITES

One leucine metabolite that has also been studied is α-KIC. As previously described α-KIC is produced from leucine by KICD (43). Albeit studies in chick cells have shown that it may supress proteolysis (292), human studies have been disappointing (293). Yarrow et al. (293), observed that when acutely supplementing RET men with either 1.5 or 9.0 g of α-KIC, no ergogenic effects were observed when comparing to placebo. A previous work, that combined a small amount (0.3 g) of α-KIC to 3 g HMB-Ca, showed reduced signs and symptoms of muscle damage, assessed by DOMS (39). However, when analysing other similar studies, it seems that this action is not driven by α-KIC. Nunan et al (294) also supplemented with 3 g HMB + 0.3 g α-KIC, during 14 days and assessed its action after a single 40 min downhill run, failing to detect any positive effects on CK, DOMS, range of motion, mid-thigh girth, isometric and concentric force.

Albeit combining α-KIC with other compounds, like glycine and arginine (GAKIC) has shown interesting results in some (295), but not all (296) studies, it is unclear whether these actions are due to α-KIC. It is unlikely that α-KIC may present any additional benefits when comparing to HMB or α-HICA.

2.3 Aim of the investigation

The present dissertation aimed to clarify and directly compare the effects of three off-the-shelf leucine metabolites, namely HMB (both as a calcium salt or as a free acid form) and α-HICA, in performance, body composition and biochemical markers of muscle damage, when consumed during a RET program.

Study 1 (Chapter 4) was performed to clarify, if supplementation with either leucine metabolite along with RET for 8 weeks in resistance trained males offered superior results towards training-induced effects on strength and power performance (1RM for bench press and squat, CMJ and Wingate test), muscle thickness (a proxy marker of skeletal muscle hypertrophy), circulating hormones (total testosterone, GH, IGF-1 and cortisol) and a proxy markers of muscle damage (CK). .

Study 2 (Chapter 5) aimed to clarify if the same supplementation protocol offers benefits over the RET program, between each leucine metabolite, on either regional and whole-body body composition outcomes, as well as the effect on TBW, intracellular water (ICW) and extracellular water (ECW).

Study 3 (Chapter 6) was designed to clarify whether any of the previously studied off-the-shelf leucine metabolites would be advantageous in preventing training-induced changes in several inflammatory markers (IL-6, hsCRP and TNF-α).

Study 4 (Chapter 7) aimed to assess the effects of supplementation with α-HICA in a type 1 diabetic patient with serious mobility limitations regarding body composition, handgrip strength, maximal knee extension strength, and blood-sampled health assessments.

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CHAPTER 3

Methodology

A brief description of the cohort will be provided in this chapter, followed by a description of all methods and procedures used throughout the investigation.

3.1 Study design and sampling

Ethics and general study design

This investigation was approved by the Institutional Review Board of the Faculty of Human Kinetics (approval number 15/2017), thus conforming with all requirements as per the declaration of Helsinki (1). Additionally, the trial was registered at clinicaltrials.gov with the number NCT03511092. Studies 1, 2 and 3 (Chapters 4, 5 and 6, respectively) emerged from a randomized double-blinded, placebo-controlled trial, while study 4 (Chapter 7) was a clinical case study investigation.

STUDY 1, 2 AND 3 (CHAPTERS 4, 5 AND 6)

Participants were between the ages of 18 and 45 years old and were recruited from social networks and local gyms. Before engaging in any procedures, subjects were informed pertaining the purpose, design and data collection methodologies of the studies. Before written and verbal consent was given, all potential risks and benefits were thoroughly explained to the participants. In these studies, individuals were subjected to supervised RET and randomly allocated to groups supplementing with either α-HICA, HMB-FA, HMB-FA or PLA. Performance (1RM, CMJ and Wingate test), body composition (DXA, US), self-reported dietary intake and blood collection assessments were all performed at baseline, end of week 4 and week 8 (Figure 15).

Participants received supplements with instructions in a double-blinded fashion. Supplementation compliance was assessed when participants delivered their empty supplement bags at the end of every 2 weeks. Performance was assessed in a fed state after the ingestion of a meal replacement bar, after muscle thickness assessments (by US), while body composition by DXA and bioimpedance spectroscopy (BIS) were performed after a 12 h fast, without prior exercise and refraining from the consumption of alcohol.

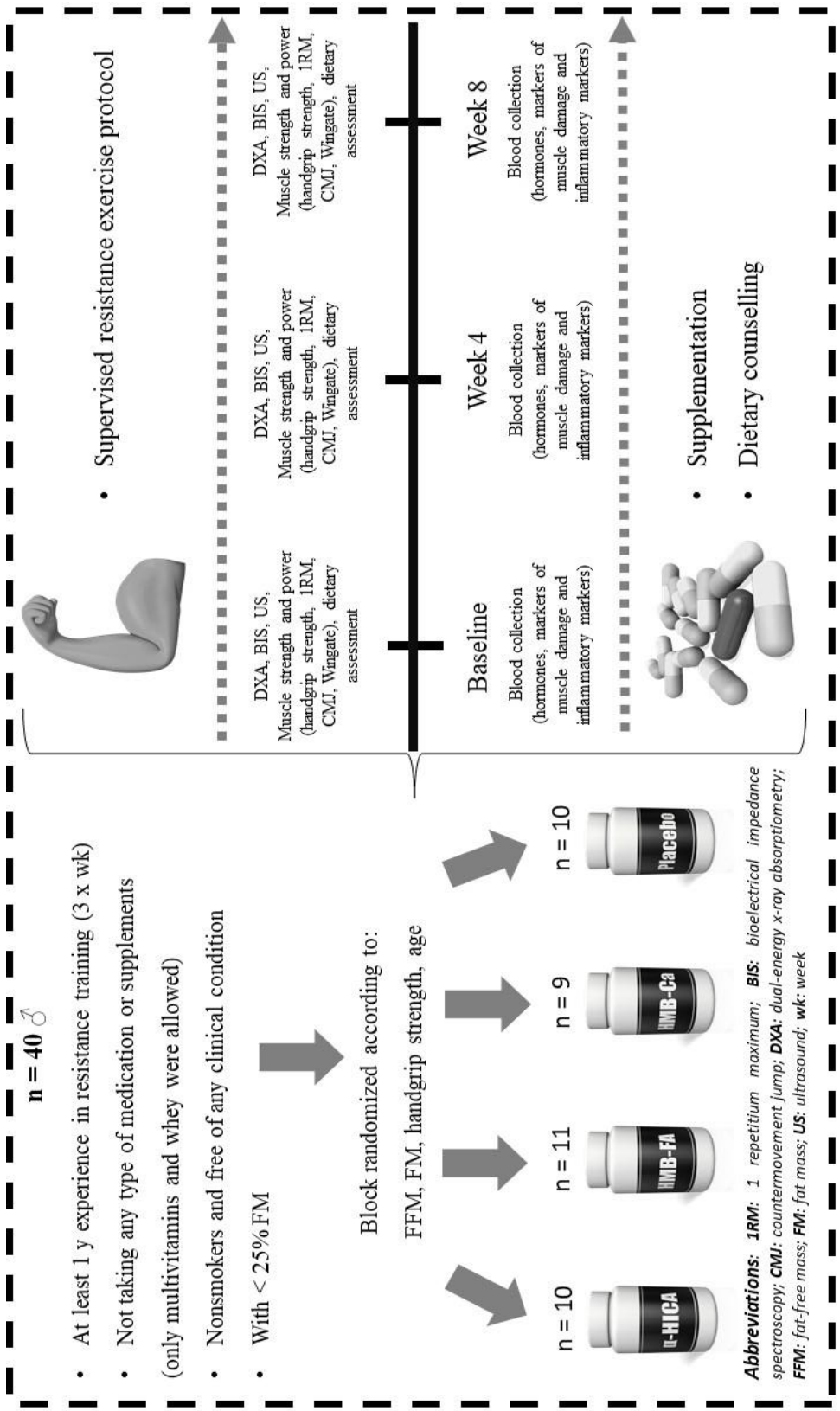

Figure 12. Research design for studies 1 (chapter 4), 2 (chapter 5) and 3 (chapter 6).

STUDY 4 (CHAPTER 7)

The participant was followed under supervised physician care. Before engaging in the research procedures, the subject was informed of all procedures, risks and benefits according to the principles set out by the declaration of Helsinki (1). The subject was evaluated at baseline and after 120 days, pertaining blood markers (prescribed by his physician), strength and body composition (DXA). Body composition measures and blood collection were performed after a 12 h fast, while a meal replacement bar war provided prior to the strength tests. No exercise protocol was prescribed (due to his mobility limitations), and the subject was instructed not to change his physical activity during the intervention. The α-HICA containing supplement was administered throughout the study, 500 mg thrice daily with main meals. Additionally, the diet was assessed at baseline and after 120 days, under the supervision of a registered dietitian.

Participants

STUDY 1, 2 AND 3 (CHAPTER 4, 5 AND 6)

Fifty-three participants were elected to participate in these studies based on two questionnaires and an interview. The first questionnaire had several questions regarding training (including training status) and dietary supplement intake. The second questionnaire was the PAR Q – Physical Activity Readiness Questionnaire to assess cardiovascular risk. Supplements were only allowed if they did not influence body composition or performance (only protein supplements and multivitamins were allowed). Volunteers undertaking any medications were automatically excluded from the study. Participants were healthy men between 18 and 45 years old, engaged in resistance training at least 3 times per week for at least 1 year.

All participants were non-smokers and free from clinical conditions that might have compromised their tolerance to the supplements, training program, or influence body composition and performance. In order to avoid obesity as a confounding factor, participants above 25% body fat were also excluded, since this is the cutoff value for obesity in men (2). Although 53 participants were initially engaged in the study, only 40

were able to complete it. Please refer to the CONSORT flow diagram for more details regarding dropouts (Figure 16).

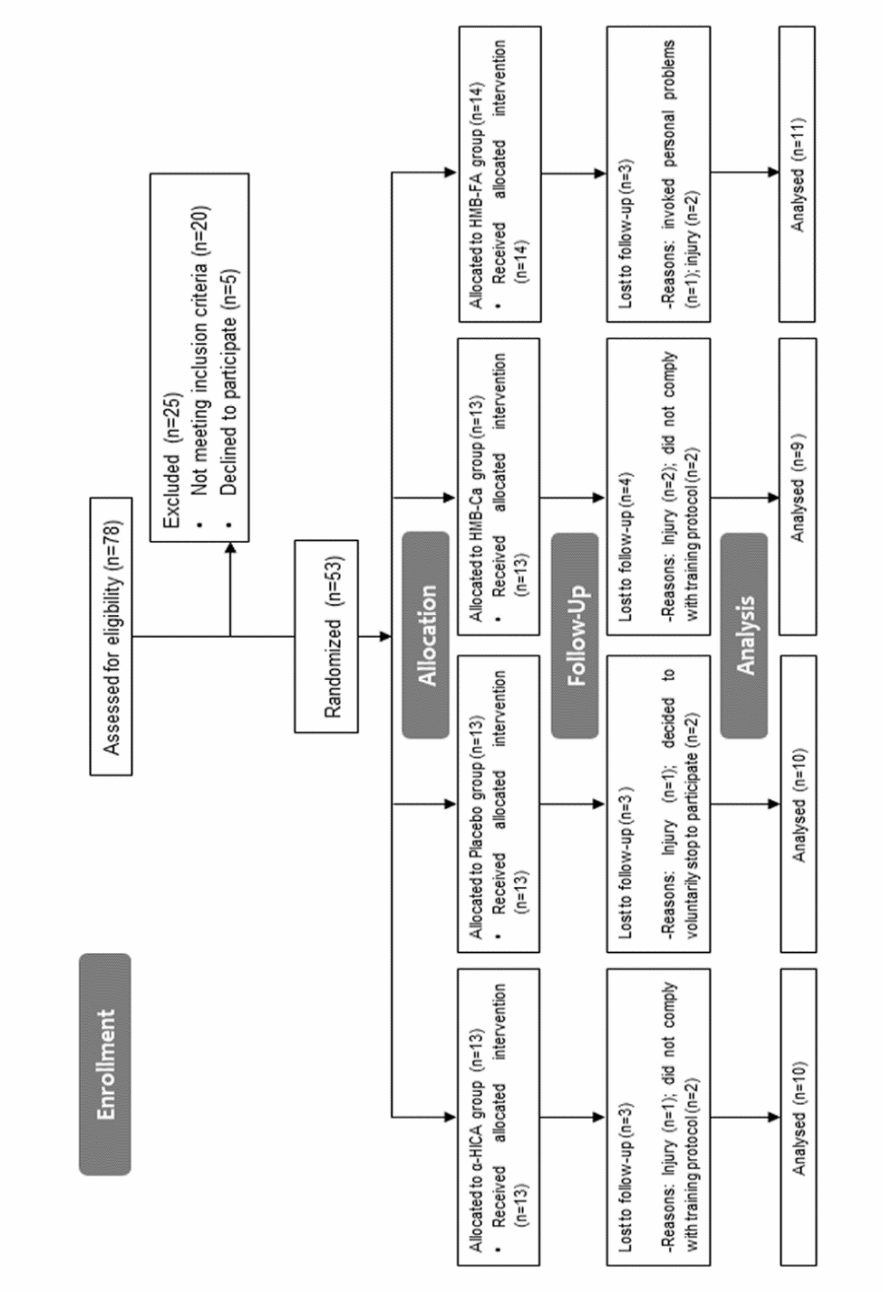

Figure 13. CONSORT flow diagram

Table 4. Baseline characteristics of the participants (n = 40)

	Age (years)	Height (cm)	Weight (kg)	FFM (kg)	FM (kg)	Handgrip dominant side (kg)
α-HICA	31 (24.7-36.7)	172 (166-177)	73.3 (66.4-80.1)	62.0 (56.9-67.1)	10.4 (7.3-13.4)	50.4 (41.5-59.3)
HMB-FA	30 (24.9-35.6)	174 (170-177)	77.0 (67.8-86.2)	62.7 (55.6-69.8)	13.3 (10.2-16.5)	51.9 (46.4-57.3)
HMB-Ca	34 (30.6-37.4)	177 (173-182)	78.2 (70.0-86.4)	65.6 (57.8-73.4)	11.6 (8.8-14.3)	55.4 (51.1-59.8)
PLA	31 (26.1-35.5)	174 (170-179)	75.8 (70.9-80.7)	64.2 (60.2-68.3)	10.7 (8.8-12.6)	50.9 (43.9-57.9)

Values are means and confidence intervals (CI) are shown in parentheses

Abbreviations: α-HICA: leucic acid**; FFM**: fat-free mass; **FM**: fat mass; **HMB-Ca**: β-hydroxy-β-methylbutyrate calcium salt; **HMB-FA**: β-hydroxy-β-methylbutyrate free acid; **PLA**: placebo

STUDY 4 (CHAPTER 7)

The volunteer was a type 1 diabetic patient (diagnosed for over 30 years) with 59 years old, non-smoking male, that was referenced to our lab and gave permission to access his medical records. According to his medical file, he was unable to engage in any type of training due to serious mobility limitations. A grand mal seizure (15 years before) led to permanent damage on his right hip, eventually requiring hip replacement surgery. The patient also presented a long history of serious hypoglycemic crises, one associated with cardiac arrest. According to the primary care physician, both the impaired ambulation and the disease were the cause for the loss of body mass, especially in the lower limbs.

The subject also used several medicines including: ≈25 IU slow acting insulin (Lantus®-Generis Farmacêutica SA, Amadora, Portugal) upon awakening and ≈3 UI fast acting insulin (Humalog®-Lilly Portugal, Lisboa, Portugal), approximately one hour after meals. The insulin administration was dependent on the glycemic values. Additional medications included pregabalin 100 mg twice daily (Lyrica® Pfizer Ltda, Freiburg, Germany) for the treatment of peripheral pain and 75 mg clopidogrel (KRKA, Cuxhaven, Germany) before sleep to prevent arterial thrombosis. Due to his mobility and medical limitations, he was always accompanied by his care taker during all assessments.

3.2 Supplementation and diet control

The assignment of the participants to the groups of supplementation was performed according to a randomly generated list and was blocked in varying block sizes according to grip strength, age and FFM. No statistically significant differences existed between groups, for these variables, at baseline (Table 4). This type of block randomization had already been performed in previous works with HMB (3-5) and has been shown to reduce bias (6).

Since several research studies have expressed concerns regarding supplements meeting the label claims (7-9), we externally assessed the supplements used, pertaining their content. All supplements, α-HICA (Onsalesit, SA, Funchal, Portugal), HMB-FA (Beta-TOR, Body Attack, Hamburg, Germany) and HMB-Ca (HMB Mega Caps 1250, Olimp Labs, Pustynia, Poland), met the label claims. According to the third party analysis using HPLC-UV (Labs-Mart Inc. Edmonton, AB – Reports: 70496-1, 70496-2 and 70496-3) the supplements contained 97 ± 2.9% of the α-HICA, 95 ± 2.3% of the HMB-FA and 96 ± 2.1 of the HMB-Ca reported. The placebo group consisted on magnesium stearate, being ordered specifically from a local manufacturer in white unlabeled capsules. Magnesium stearate is an inactive substance widely used for many decades in the food industry - mainly as an emulsifier in food supplements and herbs, spices and chewing gums (10). It also displays low genotoxic potential (10) and is categorized by the U. S. Food & Drug Administration as generally recognized as safe (GRAS) (11). Furthermore, it does not reduce other substances' bioavailability (12) and is used in clinical research as placebo (13). Each capsule had a small amount of the compound and all doses were well inside the safety limits.

In studies 1, 2 and 3 (chapter, 4, 5 and 6) HMB supplements were consumed 1 g prior to training and 1 g on every other 2 meals (total 3 x 1 g daily). On rest days HMB was consumed 1 g per main meal (total: 3 x 1 g a day). This was performed according to previous research (3, 5). The only α-HICA study performed in humans to date, used 500 mg 3 x daily with main meals (14). In order to guarantee blinding, we decided to use the same supplementation protocol with both α-HICA and HMB, since it was unlikely that the timing of ingestion would have influenced results. Different timing protocols have been investigated with leucine derivatives and no differences were reported (15-18). In

study 4 (chapter 7), since no RET was involved, α-HICA was administered 500 mg thrice daily with main meals according to Mero et al. (14). Compliance was assessed in all studies, when participants' returned their supplement empty bags during counselling (every 2 weeks - studies 1, 2, 3; every week - study 4).

Since energy intake might influence performance and body composition (19), in study 1, 2 and 3 (chapters 4, 5 and 6) the participants were individually instructed by a licensed and trained dieticians to consume sufficient energy and protein in order to allow for training-induced gains of FFM. Thus, participants self-reported 3-day dietary intake through dietary logs (3 non-consecutive days, one being a weekend day), according to Yang et al. (20), at the beginning of the study and at the end of weeks 4 and 8. Food logs were analyzed by Food Processor 10.12 (ESHA Research Inc., Salem, Oregon USA) for energy and macronutrients. Before the beginning of the study, if participants were not ingesting at least 1.6 g of protein per kg/body weight.day^{-1}, according to the most recent recommendations for muscle hypertrophy development (21), and at least 45 kcal per kg of FFM, according to the upper range for an estimated positive energy balance in athletes (19), a registered dietitian would provide dietary counselling in order to adjust the protein and/or energy intake. Eleven participants required protein adjustments, which were normalized to ≈2.2 g of protein per kg/body weight.day^{-1} (using protein supplements or food, depending on individual's convenience). This consequently raised daily mean protein intakes into a high protein approach in both groups. These strategies were used to assure that participants were in an estimated positive energy balance and that protein ingestion allowed for muscle hypertrophy development.

In study 4 (chapter 7), the volunteer followed a diet designed by a Registered Dietitian from a previous hospital admission, which was deemed adequate. He was further instructed not to change his dietary habits during the study. The diet was comprised of 2200 kcal (138 g protein ≈25% TDEE, 303 g carbohydrates ≈55% TDEE, and 49 g fat ≈ 20% TDEE) with its composition being assessed from baseline every 30 days in the same fashion as studies 1, 2 and 3 (chapters 4, 5 and 6) using certified software (Food Processor, Esha Research, Inc, Salem, Oregon, USA). Weekly Nutrition counselling was provided by a Registered Dietitian to enhance compliance with the hospital diet.

3.3 Training Protocol

Only the participants in studies 1, 2 and 3 (chapters 4, 5 and 6) were engaged in a RET protocol. The RET protocol was designed according to the guidelines for hypertrophy type of resistance training for intermediate-trained individuals and consisted of 3 sessions per week during an 8-week period, with a minimum of 48 hours interval between sessions (22-24). The following exercises were performed in the described order during each resistance training session: barbell back squat, deadlift, machine leg extension, barbell flat bench press, dumbbell military press, lat pull-down, seated cable row. During the first three weeks, participants performed three (weeks one and two) or four (week three) sets of 12 repetitions with 60-s of rest between sets and exercises. In weeks four, five and six, participants performed three (week four) or four (weeks five and six) sets of 10 repetitions with 90-s between sets and exercises. During the last two weeks (weeks seven and eight), participants performed four sets of eight repetitions with 120-s between sets and exercises (table 5).

Table 5. Characteristics of the training protocol

Weeks	Sets	Reps	Rest
1 and 2	3	12 RM	60-s
3	4	12 RM	60-s
4	3	10 RM	90-s
5 and 6	4	10 RM	90-s
7 and 8	4	8 RM	120-s

Each repetition was performed in a controlled manner for 2-s during the eccentric phase and 1-s in the concentric phase. Given that athletes had a resistance training background of at least 1 year, sets were carried to the point of concentric muscle failure while maintaining proper exercise form. All exercises were performed according to the

NSCA guidelines (25) and under the direct 1:1 supervision of an experienced strength and conditioning coach.

3.4 Muscle strength and power assessments

Before each assessment the participants were familiarized with the specific strength test. In all studies, a meal replacement bar (Matrix Bar, Olimp Labs, Pustynia, Poland) was provided prior to strength and power assessments, to both standardize dietary intake prior to tests and to guarantee participants were not in a fasted state. This bar was comprised of 26.2 g protein, 20.5 g carbohydrate, and 8 g fat.

Maximal isometric forearm strength

In all studies, handgrip strength was assessed. In studies 1, 2 and 3 (Chapters 4, 5 and 6) participants' handgrip strength was assessed at study entry and at the end of weeks 4 and 8. In study 4 (Chapter 7) this parameter was assessed at baseline and at the end of the study (after 120 days). The same procedures were enforced in all studies.

Maximal isometric forearm strength was determined using a hydraulic hand dynamometer model 5030J1 (Jamar, Sammons Preston, Inc, Bolingbrook, IL, U.S.A.) with visual feedback (26). The dynamometer was adjusted to the subject's dominant hand with each trial lasting approximately 5-seconds. The best of three maximal trials was recorded to the nearest 2 kg (19.61 N). The same adjustment of the dynamometer was used for all tests. This test was included since it has been suggested as a reliable indicator pertaining favorable prognosis in health-related events in older and diseased populations (26, 27) and also as an indicator of general strength in younger healthy adults (28).

Maximal isometric leg Strength

This assessment was only performed in study 4 (chapter 7). Since the participant presented serious mobility limitations, for safety reasons and following medical advice 1RM tests were precluded. The evaluation of the maximal lower strength was made performing 3 rapid maximal voluntary isometric contractions (MVC's). The evaluation of the maximal knee extension strength was performed on a custom-made horizontal leg

press device (Model 4090E; HBP Exclusive Line) instrumented with an aluminum platform equipped with 4 load cells (Shear Beam Load Cell - Flintec BK2). During the test, the patient was positioned with the hip and knee joints at angles of 100˚ and 110˚, respectively. The volunteer was then instructed to produce maximal force as quickly as possible, sustaining the effort for 3 seconds (29, 30). The force signal was A/D converted (MP100 – Biopac Systems Inc, 16 bits) with a sample rate of 1 KHz. AcqKnowledge software (Biopac Systems Inc) was used to analyze the highest value across the 3 MVC's to the nearest 2.0 N/mV. The same equipment adjustment was used in all assessments. This test has been proposed to have particular relevance in geriatric and rehabilitation environments, since it represents a practical and inexpensive alternative for the assessment of muscular strength and power (30).

Countermovement jump, 1 repetition maximum and Wingate test

These tests have been thoroughly used to evaluate muscle strength and power in both, young trained (3, 4) and recreationally active individuals (5), when evaluating the effects of some leucine metabolites. As explained previously, these tests were only used in studies 1, 2 and 3 (Chapters 4, 5 and 6). Before any assessments were performed, after initial familiarization with tests procedures, the subjects performed five minutes of moderate-intensity aerobic exercise (29).

Countermovement jump was assessed on a contact platform controlled by an open-source hardware and software model (Chronojump, Barcelona, Spain), which computed and stored flight time with a temporal resolution of 1 ms. The displacement of the center of gravity (jump height *h*) during flight was estimated by means of flight time through a standardized kinematic equation $h = t2.g/8$, where g is the gravity acceleration (9.81 m/s^2). The software automatically generated jump height (cm) and power (W), from the computed data. The best attempt out of 3 attempts was considered for analysis (31).

The evaluation of maximum strength was obtained from 1RM testing of the back squat and bench press exercises on a Multipower machine (model-M953, Technogym,

Cesena, Italy) at the strength laboratory at baseline and at the end of weeks 4 and 8. The determination of 1RM from these exercises was conducted according to the NSCA guidelines and supervised by a NSCA certified strength and conditioning specialist (25). In summary, the subjects performed 3 warmup sets before attempting the 1RM load. A 3-minute recovery was allowed between the last warmup set and the 1RM attempt. An increase or decrease of 2.5-5 kg on the bench press exercise and 5-10 kg on the squat exercise occurred in case the attempt to move the 1RM load was successful or failed. The order of the 1RM exercise determination was: bench press and back squat; and was maintained for the three test sessions.

Peak anaerobic power and mean anaerobic power were assessed by the Wingate anaerobic test. After initial familiarization and individual adjustment on the cycle ergometer (Monark ergomedic 894 E, Monark Exercise AB, Vansbro, Sweden), subjects performed three to five minutes of light cycling. At the end of each minute of the warm-up, the subject performed approximately five seconds of sprinting. The test was initiated with the subject pedaling at maximal cadence against no load. A verbal command provided the auditory cue to begin pedaling. Once the subject attains maximal cadence, an external load (7.5% body weight) was applied for the 30 s all-out test. The subjects remained seated during the entire test (30 s). The test was terminated after 30 s of all-out work, with participants engaging in a 2-5 min cool-down period before dismounting the cycle ergometer. Peak and average power were calculated according to the manufacturer's anaerobic test software from: $Peak\ Power = (flywheel\ rpm\ for\ the\ highest\ five\ second\ period\ X\ 1.615\ m)\ X\ (resistance\ (kg)\ X\ 9.8)$; $Mean\ Power = average\ of\ all\ five\ second\ intervals\ through\ the\ entire\ 30\ s\ test$

These tests were used due to their high reliability regarding muscle power and strength. 1RM tests have been recommended to assess lower body and upper body strength, while the 30 s Wingate anaerobic test has been used as a surrogate for anaerobic endurance, capacity and fatigue (29). Insofar as jump performance is concerned, CMJ was used instead of squat jump, since the former seems superior to assess athletic performance (32).

3.5 Body composition measurements

Anthropometry

All subjects were measured to the nearest 0.1 cm with a stadiometer (Seca, Hamburg, Germany), using standardized procedures (33). Body mass was assessed to the nearest 0.1 kg using a weight scale (Seca, Hamburg, Germany). Participants in studies 1, 2 and 3 (Chapters 4, 5 and 6) were assessed at baseline and after weeks 4 and 8. The participant in study 4 (chapter 7), was assessed at baseline and after 120 days.

Bioelectrical impedance spectroscopy

BIS was used in study 2 (Chapter 5) to assess TBW from ECW and ICW. The ECW and ICW compartments were determined by whole body resistance (R) and reactance (Xc) and TBW calculated as their sum, using BIS model 4200 from Xitron Technologies (San Diego, CA, USA).

Participants were evaluated in a supine position with their arms and legs abducted at an angle of 45º. After the skin was cleaned with alcohol, four electrodes were placed on the dorsal surfaces of the right hand and foot. The source electrodes were placed on the hand, in the middle of the dorsal surface proximal to the metacarpal-phalangeal joint, and on the foot, in the middle of the dorsal surface proximal to the metatarsal-phalangeal joint. The detector electrodes were placed on the wrist at the midline between the distal prominences of the radius and ulna and in the ankle joint at the line between the malleoli. Before each testing session, the analyser was calibrated with a calibration circuit simulator.

Assessments were performed after a 10-minute rest period (34). The software performed biophysical modelling according to the impedance data (from R and Xc). Extracellular water and intracellular water were calculated individually using the equipment's prediction equations. Total body water was assumed as the sum of ECW and ICW. This method has been previously validated to estimate total body water and its compartments in athletes (34).

Dual-Energy X-Ray Absorptiometry

In all studies, participants underwent a whole-body DXA scan on a Hologic Explorer-W, fan-beam densitometer (Hologic, Waltham, MA) according to the procedures recommended by the manufacturer. Studies 2 and 4 (Chapters 5 and 7) analyzed whole-body and regional body composition. Study 2 analyzed the changes between baseline and weeks 4 or 8, while study 4 performed the same analysis between baseline and day 120 (end of the study). Whole-body FFM, FM and BMC were analyzed only at baseline in studies 1 and 3, to allow block randomization.

The DXA equipment measures the attenuation of X-rays pulsed between 70 and 140 kV synchronously with the line frequency for each pixel of the scanned image. A step phantom with six fields of acrylic and aluminum of varying thickness and known absorptive properties was used as an external standard calibrator for the analysis of different tissue components.

Although the software automatically generates regions (i.e. arms, legs and trunk), those were adjusted manually by the technician. Specifically, trunk region was separated from the legs by a horizontal line right above the iliac crest (lower boundary) and from the head by neck cut (upper boundary) (35). The trunk region includes the neck, chest, abdominal and pelvic areas except the gluteal area that was included into legs (36).

The same technician positioned the participant, performed the scan, and executed the analyses (software QDR for Windows version 12.4; Hologic) according to the operators manual using the standard analysis protocol. Analysis provided total FM, FFM and BMC from whole-body and different subregions. The coefficients of variation in our laboratory, based on 10 young active adults (five males and five females), is 1.6% for bone mineral content, 1.7% for FM, and 0.8% for FFM (37). This method was used due to its good precision and low radiation dose (38), although changes in hydration may be a concern (39, 40). Additionally, DXA offers regional body analysis and is presently considered the gold standard for BMC assessment (38-41).

Muscle Thickness

Muscle thickness was only assessed at baseline and at the end of weeks 4 and 8 in studies 1 and 3 (Chapters 4 and 6). Muscle thickness of the vastus lateralis (VL) and the rectus femoris (RF) was assessed at rest using B-mode ultrasound imaging with a 9-cm-long 10-MHz linear-array transducer (model EUB-7500; Hitachi Medical Corporation, Tokyo, Japan). Longitudinal and transversal scans were taken from the muscles mid-belly corresponding to 39% (VL) and 56% (RF) of the distance from the proximal edge of the patella to the anterior superior iliac spine, according to Blazevich et al. (42).

Participants were positioned in a seated position with their knee flexed at 10° (0° being full extension), participant's legs were supported during the scan and their muscles relaxed. To ensure that repeated scans (weeks 4 and 8) were taken from the same site, scanning locations were mapped with a malleable transparent plastic sheet at the baseline measurement, along with other distinguishing surface landmarks (e.g., border of patella, tattoos, scars, moles). We defined MT as the perpendicular distance between the subcutaneous adipose tissue-muscle interface and intermuscular interface, and quantified three times from the ultrasound scans using the image analysis software, IMAGEJ 1.42q (National Institutes of Health, Bethesda, MD). Averaged values were considered for further analysis. All measures were collected and digitally analyzed by the same operator with an intra-rater coefficient of variation of 0.5% for VL and 0.6% for RF.

This method has been used as a proxy marker to assess skeletal muscle hypertrophy in previous studies (3, 4) and has been shown to correlate well against more robust and direct measures of muscle mass (43, 44).

3.6 Blood markers

Hormones and proxy markers of muscle damage

Hormones and proxy markers of muscle damage were evaluated in study 1 (Chapter 4). Blood samples were collected by standard procedures into ethylenediaminetetraacetic acid (EDTA) tubes, centrifuged at 500 g at 4 °C for 15 min, and plasma was frozen at -80 °C. Plasma samples were then analyzed for GH, total

testosterone, IGF-1 and cortisol at the Core Laboratory of McMaster University Medical Centre using solid-phase, two site chemiluminescence immunometric assays (Immulite; Intermedico, Holliston, MA).

All intra-assay coefficients of variation for these markers were below 5% and all assays included external and internal standards and daily quality controls. Plasma creatine kinase (CK) was determined by a kit according to the manufacturers specifications (Sigma Aldrich, St. Louis, MO). The intra-assay coefficient of variation (CV) for CK was below 8%. Hemoglobin was analyzed by photometry (Beckman, DU68) by the Drabkin method (45) and hematocrit by capillary microcentrifugation (Sigma Aldrich, St. Louis, MO). These markers have been used previously in several studies, with leucine metabolites, to evaluate both endocrine and muscle damage effects of these compounds (3, 15, 46-48).

Inflammatory markers

Inflammatory markers were only assessed in study 3 (Chapter 6). Blood collection was performed within the same standards as for hormones and proxy markers of muscle damage. Plasma was then further analysed for IL-6, high-sensitivity C-reactive protein (hsCRP) and TNF-α concentrations also at the core Laboratory of McMaster University Medical Centre. IL-6 and TNF-α were analysed using a Bio-Plex reagent Kit and a Bio-Plex reader (Bio-Rad Laboratories, Hercules, CA) by enzyme-linked immunosorbent assay (ELISA), while hsCRP was analysed using a commercially available high-sensitivity CRP-Latex Kit (Pulse Scientific, Burlington, ON, CA) and an Express Plus autoanalyzer (Chiron Diagnostics Co, Walpole, MA). Intra-assay coefficients of variation reported for the lab are <6%, <4.5% and <3.5%, for IL-6, TNF-α and hsCRP, respectively.

At weeks 4 and 8, concentrations of hormones, inflammatory and muscle damage markers, were corrected for plasma volume variation with hemoglobin concentration and hematocrit, according to Dill & Costill (49) (studies 1 and 3).

Blood markers requested under physician care

In study 4 (Chapter 7), several biomedical markers were requested under physician order due to safety reasons. These included: Hemoglobin, Erythrocytes, Hematocrit, Mean Corpuscular Volume, Red Cell Distribution Width, Platelet Count, Ferritin, Transferrin, Serum Iron, Glucose, Glycated Hemoglobin, Median Glucose, Insulin, Total Cholesterol, low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), Triglycerides, Albumin, C Reactive Protein, Uricemia, Creatinine, Aspartate Aminotransferase, Alanine Aminotransferase, Gamma-glutamyl Transpeptidase, Alkaline Phosphatase, Sodium, Potassium, Chlorine and Free Testosterone. Blood samples were taken at a local hospital (CUF/José de Mello Saúde, Sintra, Portugal) and processed onsite in a certified laboratory.

3.7 Statistical analysis

Data analysis was performed using the IBM SPSS Statistics (SPSS Inc., an IBM Company, Chicago, Illinois, USA) version 22.0 for studies 1, 2 and 3 (Chapters 4, 5 and 6). Sample sizes for studies 1 to 3 were calculated through an a priori power analysis (G*Power Version 3.1.9.2, Heinrich Heine Universitat Dusseldorf, Germany), based on FFM changes from previous studies (4) and power of 0.80 and alpha of 0.05. No statistical analysis was performed in study 4 (Chapter 7) since this was a single case study (n = 1).

The statistical procedures common to all studies (Chapter 4 to 6) are presented below:

- Descriptive statistics including means, standard deviation and confidence interval were performed for all outcome measurements. The normality of the distribution of variables was tested by the Shapiro–Wilk test.
- Baseline values between groups and delta values were analysed by one-way ANOVA, since normality was observed.
- Time and time-by-group interactions were evaluated by repeated-measures ANOVA (Baseline, weeks 4 and 8).
- Post-hoc analysis using the Bonferroni test were used, when appropriate.

- The equality of the matrix of variance and sphericity were explored with the Levene F test and Mauchly's test, respectively.
- Overall significance level for α was set at $P \leq 0.05$.

Additionally in study 3 (Chapter 6), backwards elimination regression equations were generated in effort to elucidate if a combination of inflammatory markers shared variance with any primary outcomes (Δ% combined 1RM, Δ% VL MT, and Δ% RF MT) or baseline values (combined 1RM, VL MT, and RF MT), initial, late and total % changes. The probability of F was used as our stepwise criteria with entry at 0.05 and removal at 0.06. Scatter plots with *ZRESID plotted against *ZPRED were used to assess linearity and heteroscedasticity when one or more independent variables were retained in the model.

The specific procedures and details may be found in each chapter.

3.8 References

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CHAPTER 4

Leucine Metabolites Do Not Enhance Training-induced Performance or Muscle Thickness[1]

[1]Teixeira FJ, Matias CN, Monteiro CP, Valamatos MJ, Reis JF, Tavares F, Batista A, Domingos C, Alves F, *Sardinha LB, Phillips SM. Leucine Metabolites Do Not Enhance Training-induced Performance or Muscle Thickness. Medicine & Science in Sports & Exercise. 2019;51(1):56-64. doi:10.1249/MSS.0000000000001754*

Leucine Metabolites Do Not Enhance Training-induced Performance or Muscle Thickness

Filipe J. Teixeira, Catarina N. Matias, Cristina P. Monteiro, Maria J. Valamatos, Joana F. Reis, Francisco Tavares, Ana Batista, Christophe Domingos, Francisco Alves, Luís B. Sardinha and Stuart M. Phillips

Abstract

Leucine metabolites, α-hydroxyisocaproic acid (α-HICA) and β-hydroxy-β-methylbutyrate (calcium, HMB-Ca and free acid, HMB-FA), have been proposed to augment resistance training-induced changes in body composition and performance. **PURPOSE**: We aimed to conduct a double-blind randomized controlled pragmatic trial to evaluate the effects of off-the-shelf leucine metabolite supplements of α-HICA, HMB-FA and HMB-Ca, on resistance training-induced changes in muscle thickness, and performance. **METHODS**: Forty men were randomly assigned to receive α-HICA (n=10, fat-free mass [FFM]=62.0 ± 7.1 kg), HMB-FA (n=11, FFM=62.7 ± 10.5 kg), HMB-Ca (n=9, FFM=65.6 ± 10.1 kg), or placebo (PLA; n=10, FFM=64.2 ± 5.7 kg). The training program consisted of whole body thrice weekly resistance training for 8wk (7 exercises/session, 3-4 sets per session, at 70-80% 1RM). Skeletal muscle thickness by ultrasound, performance measures, and blood measures (creatine kinase [CK], insulin-like growth factor 1 [IGF-1], growth hormone [GH], cortisol and total testosterone) were evaluated at baseline and at the end of weeks 4 and 8. **RESULTS:** Time-dependent changes were observed for muscle thickness ($p < 0.001$), 1RM bench press and squat ($p < 0.001$), Wingate peak power ($p = 0.02$), countermovement jump height ($p = 0.03$), power ($p = 0.006$), CK, IGF-1, GH, and cortisol (all $p < 0.001$). No significant between-group or time-by-group interactions were observed. **CONCLUSION:** No leucine metabolite resulted in any ergogenic effects on any outcome variable. Supplementation with leucine metabolites – α-HICA, HMB-FA, or HMB-Ca – is not a supplementation strategy that improves muscle growth and strength development in young adult men.

Keywords: *resistance training; hypertrophy; strength; β-hydroxy β-methylbutyrate, α-hydroxyisocaproic acid*

4.1 Introduction

Leucine is an essential branched-chain amino acid present in a number of protein-containing foods (1). Leucine metabolites such as α-hydroxyisocaproic acid (α-HICA) and β-hydroxy-β-methylbutyrate (HMB) have been proposed to promote changes in body composition, performance and reduce indirect markers of muscle damage (2, 3). A product of leucine metabolism, α-HICA (leucic acid) is formed in many human tissues (4). Research on α-HICA is scarce, with only one study (3) in male soccer players. Based on this single investigation (3), it has been suggested that α-HICA may exert an anti-catabolic action.

Supplementation with the leucine metabolite β-hydroxy-β-methylbutyrate (HMB) in the calcium form, HMB-Ca, has shown positive effects on performance and body composition (5-8) while other studies report no effect (9-11). The suggestions have been that the most effective use of HMB would be in older adults, untrained individuals, and injured or energy-restricted athletes (12). A free acid (FA) form of HMB (HMB-FA) has a higher bioavailability when comparing with HMB-Ca (13); however, there is no difference between HMB-Ca and HMB-FA in their ability to enhance MPS in young men (14). A recent systematic review suggested that HMB-FA, in conjunction with resistance training, may attenuate markers of muscle damage, augment acute endocrine responses, and enhance training-induced increases in muscle mass and strength (2). However, the reported effects on muscle mass and strength in that review are strongly driven by two research studies, which contained extraordinary results (15, 16) that have been questioned due to extensive limitations (17). Thus, it is unclear at this time as to the true efficacy of HMB-FA in promoting changes in body composition.

Meta-analyses indicate small effects of HMB on markers of muscle damage, endocrine responses and training induced changes in muscle mass and strength (2), while other analyses do not support this conclusion (18, 19). To our knowledge, there has been no randomized trial to investigate the effects of commercially available forms of these compounds on muscle thickness and selected performance outcomes. This is, in our view, exceptionally important as consumers are taking these supplements direct 'from the shelf' often based on claims stemming from research. Thus, we undertook a pragmatic controlled, double-blind randomized trial to compare commercially available

supplements, HMB-FA, α-HICA and HMB-Ca, on resistance training-induced changes in muscle thickness and performance. Our working hypothesis was that so long as participants adhered to a diet containing adequate energy and dietary protein that there would be no differences between those receiving the leucine metabolites – HMB-FA, α-HICA and HMB-Ca – when compared to a placebo consuming group.

4.2 Methods

ETHICS AND GENERAL STUDY DESIGN

This investigation was approved by the Faculty of Human Kinetics Institutional Review Board (approval number 15/2017) and conformed to all standards of human research set out in the declaration of Helsinki. The trial was registered at clinicaltrials.gov as NCT03511092. Prior to engaging in any of the study procedures, the purpose and design of the study, the data collection methodologies and all potential risks and benefits were explained to potential research participants. All participants gave their verbal and written informed consent before enrolling. Fifty-three men were recruited according to the eligibility criteria with forty completing the investigation. Participants were between the ages of 18 and 45 y and were recruited from social networks and local gyms. Participants were randomly assigned to one of the four groups: α-HICA; HMB-FA; HMB-Ca; Placebo (PLA). For details, please refer to the CONSORT flow diagram (Figure 17).

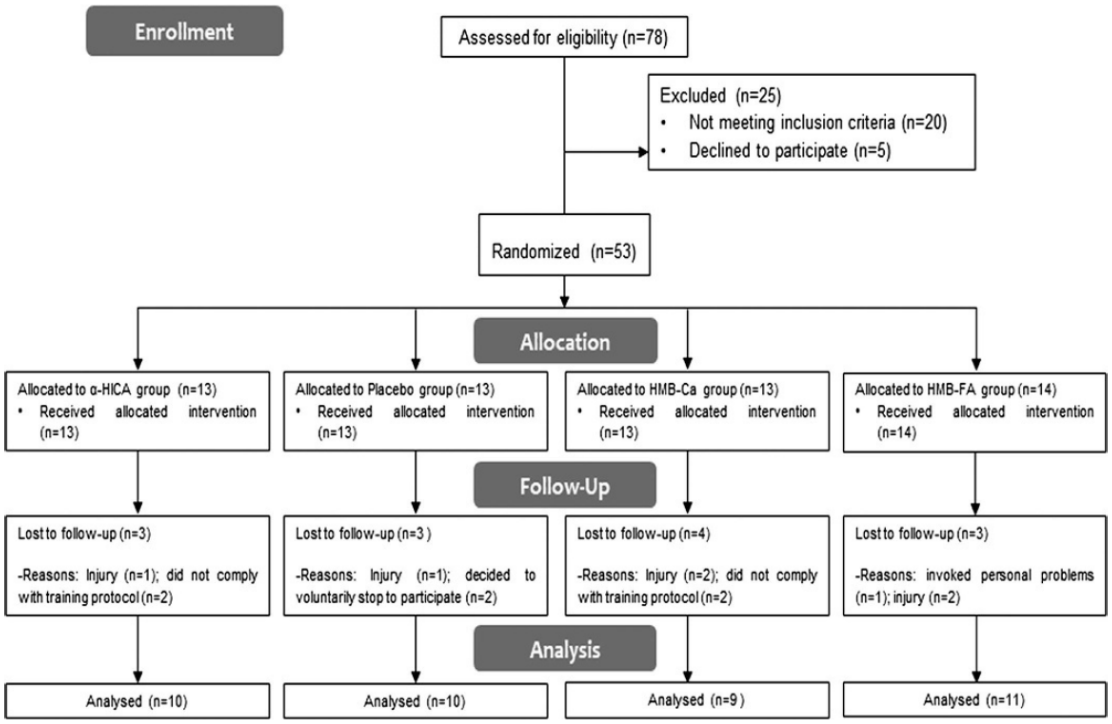

Figure 14. CONSORT diagram of the randomization and flow of participants through the study.

All supplements were generously donated directly from shelf-stock from a local supplement store. All brands presented a certificate of analysis regarding the content of each supplements. Third party testing (Labs-Mart Inc. Edmonton, AB) of the same supplements (reports 70496-1, 70496-2 and 70496-3) for α-HICA, HMB-FA and HMB-Ca (using HPLC-UV) indicated the supplements contained 97 ± 2.9% of the α-HICA, 95 ± 2.3% of the HMB-FA and 96 ± 2.1 of the HMB-Ca reported. Placebo consisted on magnesium stearate. Assignment was according to a randomly generated list and was blocked in varying block sizes with participants matched for grip strength, age and DXA-measured fat-free mass (FFM). Thus, at baseline there were no statistically significant differences between groups for handgrip strength, age or FFM (Table 6). Participants received packaged supplements and instructions in a double blinded fashion. Supplementation compliance was assessed by having the participants hand their empty supplement packets back to the researchers at the end of each two-week block of training. The training protocol consisted on whole body hypertrophy-type resistance training routine for intermediate-trained individuals and consisted of 3 training sessions per week for 8 weeks.

Evaluations of strength, skeletal muscle and performance took place at baseline and weeks 4 and 8. In addition, each subject had a blood sample collected in the same time in the morning by the same technician and were repeated at the end of week 4 and 8 of the study. All evaluations of the eligible participants and blood samples collection were performed early in the morning after a 12-h fast and without prior exercise, or consumption of alcohol, or caffeine/stimulant beverages. Muscle strength and power were assessed in a fed state after the ingestion of a meal replacement bar (Matrix Bar, Olimp Labs, Pustynia, Poland: comprised of 26.2 g protein, 20.5 g carbohydrate, and 8 g fat.

PARTICIPANTS

Participants were healthy men between 18 and 45 years old, currently engaged in resistance training for at least 1 year and at least 3 times per week. Sample size was calculated from previous studies (please refer to statistics section for more details). Participants taking any type of medication or supplements aimed at enhancing body composition or performance prior to the research, were excluded (only protein supplements and multivitamins were allowed). All participants were non-smokers and free from clinical conditions that might have compromised their tolerance of the supplements, training program, or influence body composition and performance. Participants with more than 25% body fat were also excluded.

Table 6. Baseline characteristics of the participants.

	Age (yr)	Height (cm)	Weight (kg)	FFM (kg)	FM (kg)	Handgrip Dominant Side (kg)
α-HICA	31 (24.7–36.7)	172 (166–177)	73.3 (66.4–80.1)	62.0 (56.9–67.1)	10.4 (7.3–13.4)	50.4 (41.5–59.3)
HMB-FA	30 (24.9–35.6)	174 (170–177)	77.0 (67.8–86.2)	62.7 (55.6–69.8)	13.3 (10.2–16.5)	51.9 (46.4–57.3)
HMB-Ca	34 (30.6–37.4)	177 (173–182)	78.2 (70.0–86.4)	65.6 (57.8–73.4)	11.6 (8.8–14.3)	55.4 (51.1–59.8)
PLA	31 (26.1–35.5)	174 (170–179)	75.8 (70.9–80.7)	64.2 (60.2–68.3)	10.7 (8.8–12.6)	50.9 (43.9–57.9)

Values are means, and CI are shown in parentheses.

BODY COMPOSITION

Body composition was determined at baseline by dual-energy x-ray absorptiometry (DXA) and muscle thickness by ultrasonography. Participants underwent a whole-body DXA scan according to the procedures recommended by the manufacturer on a Hologic Explorer-W, fan-beam densitometer (Hologic, Waltham, Massachusetts, USA). The equipment measures the attenuation of X-rays pulsed between 70 and 140 kV synchronously with the line frequency for each pixel of the scanned image. A step phantom with six fields of acrylic and aluminum of varying thickness and known

absorptive properties was used as an external standard calibrator for the analysis of different tissue components. The same technician positioned the patient, performed the scan, and executed the analyses (software QDR for Windows version 12.4, Hologic, Waltham, Massachusetts, USA) according to the operator's manual using the standard analysis protocol. The coefficients of variation in our laboratory, based on 10 young active adults (five males and five females), is 1.6% for BMC, 1.7% for FM, and 0.8% for FFM (20).

MUSCLE THICKNESS

Muscle thickness (MT) of the vastus lateralis (VL) and the rectus femoris (RF) was assessed at rest using B-mode ultrasound imaging with a 9-cm-long 10 MHz linear-array transducer (model EUB-7500, Hitachi Medical Corporation, Tokyo, Japan). Longitudinal and transversal scans were taken from the muscles mid-belly corresponding to 39% (VL) and 56% (RF) of the distance from the proximal edge of the patella to the anterior superior iliac spine, according to Blazevich et al. (21). Participants were positioned in a seated position with their knee flexed at 10° (0° being full extension), participant's legs were supported during the scan and their muscles relaxed. To ensure that repeated scans (weeks 4 and 8) were taken from the same site, scanning locations were mapped with a malleable transparent plastic sheet at the baseline measurement, along with other distinguishing surface landmarks (e.g., border of patella, tattoos, scars, moles). We defined MT as the perpendicular distance between the subcutaneous adipose tissue-muscle interface and intermuscular interface, and quantified three times from the ultrasound scans using the image analysis software, IMAGEJ 1.42q (National Institutes of Health, Bethesda, MD). Averaged values were considered for further analysis. All measures were collected and digitally analyzed by the same operator with an intra-rater coefficient of variation (CV) of 0.5% for VL and 0.6% for RF.

BLOOD MARKERS

Blood samples were collected by standard procedures into EDTA tubes, centrifuged at 500 g at 4 °C for 15 min, and plasma was frozen at -80 °C. Plasma samples were then analyzed for growth hormone (GH), total testosterone, insulin-like growth factor 1 (IGF-1) and cortisol at the Core Laboratory of McMaster University Medical Centre using solid-phase, two site chemiluminescence immunometric assays (Immulite;

Intermedico, Holliston, MA). All intra-assay coefficients of variation for these markers were below 5% and all assays included external and internal standards and daily quality controls. Plasma creatine kinase (CK) was determined by a kit according to the manufacturers specifications (Sigma Aldrich, St. Louis, MO). The intra-assay CV for CK was below 8%. Hemoglobin was analyzed by photometry (Beckman, DU68) by the Drabkin method and hematocrit by capillary microcentrifugation (Sigma Aldrich, St. Louis, MO). Concentrations of hormones and metabolites were corrected for plasma volume variation with hemoglobin concentration and hematocrit.

MUSCLE STRENGTH AND POWER

Muscle strength was assessed at study entry by evaluating grip strength and 1 repetition maximum (1 RM) of the back squat and bench press at baseline and at the end of weeks 4 and 8. Maximal isometric forearm strength was determined using a handgrip dynamometer (Jamar, Sammons Preston, Inc, Bolingbrook, IL). The evaluation of maximum strength during the protocol was obtained from 1-RM testing of the back squat and bench press exercises on a Multipower machine (model-M953, Technogym, Cesena, Italy). The determination of 1-RM from these exercises was conducted according to the National Strength and Conditioning Association (NSCA) guidelines and supervised by an NSCA-certified strength and conditioning specialist.

Muscle power was assessed with a supramaximal cycling test (Wingate) and a countermovement jump (CMJ). During the Wingate test (using a cycle ergometer – Monark ergomedic 894 E, Monark Exercise AB, Vansbro, Sweden), volunteers were instructed to cycle against a predetermined resistance (7.5% body weight) as fast as possible for 30 seconds (22). Peak power and average power were calculated. Countermovement jump was assessed on a contact platform controlled by an open-source hardware and software model (Chronojump, Barcelona, Spain), which computed and stored flight time with a temporal resolution of 1 ms. The best attempt out of 3 was considered for analysis (23).

SUPPLEMENTATION AND DIET CONTROL

Each participant received a commercial form of either α-HICA (HICA, Onsalesit, SA, Funchal, Portugal), HMB-FA (Beta-TOR, Body Attack, Hamburg, Germany), HMB-Ca (HMB Mega Caps 1250, Olimp Labs, Pustynia, Poland), or placebo (magnesium

stearate, EightJuice, Seixal, Portugal). Participants were only aware that these were leucine derivatives and that a placebo group existed. Compounds were distributed in a double-blind manner. The investigator responsible for the sample randomization and compound distribution was not directly involved in participants' eligibility interview or data collection. Individuals ingested supplements or placebo three times daily, alongside with meals or prior to training, according with previous research: 3 x 500 mg for α-HICA (3) and 3 x 1 g for HMB-FA (16), HMB-Ca (24) or placebo.

Participants were individually instructed by licensed and trained dieticians to consume sufficient energy and protein in order to allow for training-induced gains of lean mass. Participants self-reported three-day dietary intake through dietary logs at the beginning, fourth and last week of the study. Food logs were analyzed by Food Processor 10.12 (ESHA Research Inc., Salem, Oregon USA) for energy and macronutrients. Before the beginning of the study, if participants were not ingesting at least 1.6 $g.kg^{-1}.d^{-1}$ protein per body weight (25) and at least 45 $kcal.kg^{-1}$ of FFM a day (26), a registered dietitian would provide counselling on foods to consume in order to adjust the protein and/or energy intake (Table 7). Eleven participants required protein adjustments, which were normalized to ≈2.2 g of protein per kg/body $weight.day^{-1}$ (using protein supplements or food, depending on individual's convenience). This consequently raised daily mean protein intakes into a high protein approach in all groups. These strategies were used to assure that participants were in an estimated positive

Table 7. Baseline participants' reported dietary intakes.

	Total Energy (kcal)	Energy (kcal·kg^{-1} BW)	Energy (kcal·kg^{-1} FFM)	Total Protein (g)	Protein (g·kg^{-1}BW)	Total Carbohydrates (g)	Total Fat (g)
α-HICA	3176 ± 414	44.1 ± 8.8	51.9 ± 9.3	238 ± 31	3.31 ± 0.7	397 ± 52	69 ± 13
HMB-FA	3011 ± 463	39.3 ± 2.8	48.3 ± 3.3	226 ± 35	2.95 ± 0.2	376 ± 58	67 ± 10
HMB-Ca	3384 ± 740	43.5 ± 9.3	51.8 ± 9.8	254 ± 56	3.26 ± 0.7	423 ± 92	75 ± 17
PLA	3104 ± 580	40.9 ± 6.9	48.4 ± 8.8	233 ± 44	3.07 ± 0.5	388 ± 73	69 ± 13

Values are mean±SD.

TRAINING AND EXERCISE PROTOCOLS

The resistance training protocol was designed according to the guidelines for hypertrophy type of resistance training for intermediate-trained individuals and consisted of 3 sessions per week during an 8-week period, with a minimum of 48 hours interval between sessions (27). The following exercises were performed in the described order during each resistance training session: barbell back squat, deadlift, machine leg

extension, barbell flat bench press, dumbbell military press, lat pull-down, seated cable row. During the first three weeks, participants performed three (weeks one and two) or four (week three) sets of 12 repetitions with 60-s of rest between sets and exercises. In weeks four, five and six, participants performed three (week four) or four (weeks five and six) sets of 10 repetitions with 90-s between sets and exercises. During the last two weeks (weeks seven and eight), participants performed four sets of eight repetitions with 120-s between sets and exercises.

Each repetition was performed in a controlled manner for 2-s during the eccentric phase and 1-s in the concentric phase. Given that athletes had a resistance training background of at least 1 year, sets were carried to the point of concentric muscle failure while maintaining proper exercise form. All exercises were performed according to the NSCA guidelines and under the direct 1:1 supervision of an experienced strength and conditioning coach.

STATISTICS

Sample size was calculated through an a priori power analysis (G*Power Version 3.1.9.2, Heinrich Heine Universitat Dusseldorf, Germany), based on FFM changes from previous studies (15) and power of 0.80 and alpha of 0.05. Statistical analysis was performed using IBM SPSS statistics version 22.0 (IBM, Chicago, Illinois, USA). Normality of the distribution of variables was tested by Shapiro-Wilk test. Baseline characteristics between groups and delta from baseline to week 8 for MT of both VL and RF were analyzed by a 1-way analysis of variance (ANOVA), since normality was observed. Time and time-by-group interactions were evaluated by repeated-measures ANOVA. The equality of the matrix of variance and sphericity were explored with the Levene F test and Mauchlys test, respectively. Overall significance level for α was set at $p \leq 0.05$.

4.3 Results

According to food records, there were no differences between groups at baseline and no differences occurred from baseline to the end of the study in dietary intake. Participants were compliant with the supplementation, taking 84 ± 1% of supplements with no adverse effects being reported at the end of the 4^{th} or 8^{th} week of the study.

Participants completed 94 ± 5% (α-HICA: 96 ± 4%; HMB-FA: 92 ± 5%; HMB-Ca: 96 ± 5%; PLA: 95 ± 6%) of the prescribed training sessions during the study.

BODY COMPOSITION

Muscle thickness increased for VL by 8% (95% CI: 2.5-13.6; α-HICA), 6% (95% CI: 3.5-8.6; HMB-FA), 9% (95% CI: 1.7-15.8; HMB-Ca) and 8% (95% CI: 2.17-13.1; PLA) and for RF by 3% (95% CI: -3.1-8.9; α-HICA), 4% (95% CI: 1.7-6.1; HMB-FA), 6% (95% CI: 1.0-10.3; HMB-Ca) and 8% (95% CI: 3.2-13.4; PLA), from baseline to week 4 (VL and RF: $p < 0.001$) and from week 4 to week 8 (VL: $p = 0.009$; RF: $p = 0.018$), with no difference between groups regarding delta changes from baseline to week 8 (Figure 18).

BLOOD MARKERS

Plasma CK, IGF-1, total cortisol and GH increased from baseline to week 4 ($p < 0.001$) with no differences from week 4 to week 8 (Figure 19). There was no change in total testosterone.

MUSCLE STRENGTH AND POWER

A time effect was found for Wingate peak power ($p = 0.018$), CMJ height ($p = 0.028$), CMJ power ($p = 0.006$), and 1 RM back squat and bench press ($p < 0.001$). Wingate peak power decreased from baseline to week 4 ($p = 0.007$) returning to baseline values at the end of week 8; both CMJ height and power increased from baseline to week 8 (CMJ height: $p = 0.043$; CMJ power: $p = 0.007$); and 1RM back squat and bench press increased from baseline to week 4 ($p < 0.001$) and remained elevated at week 8 in both exercises ($p < 0.001$ and $p = 0.001$, respectively) (Table 8). There were no differences in these outcomes by group.

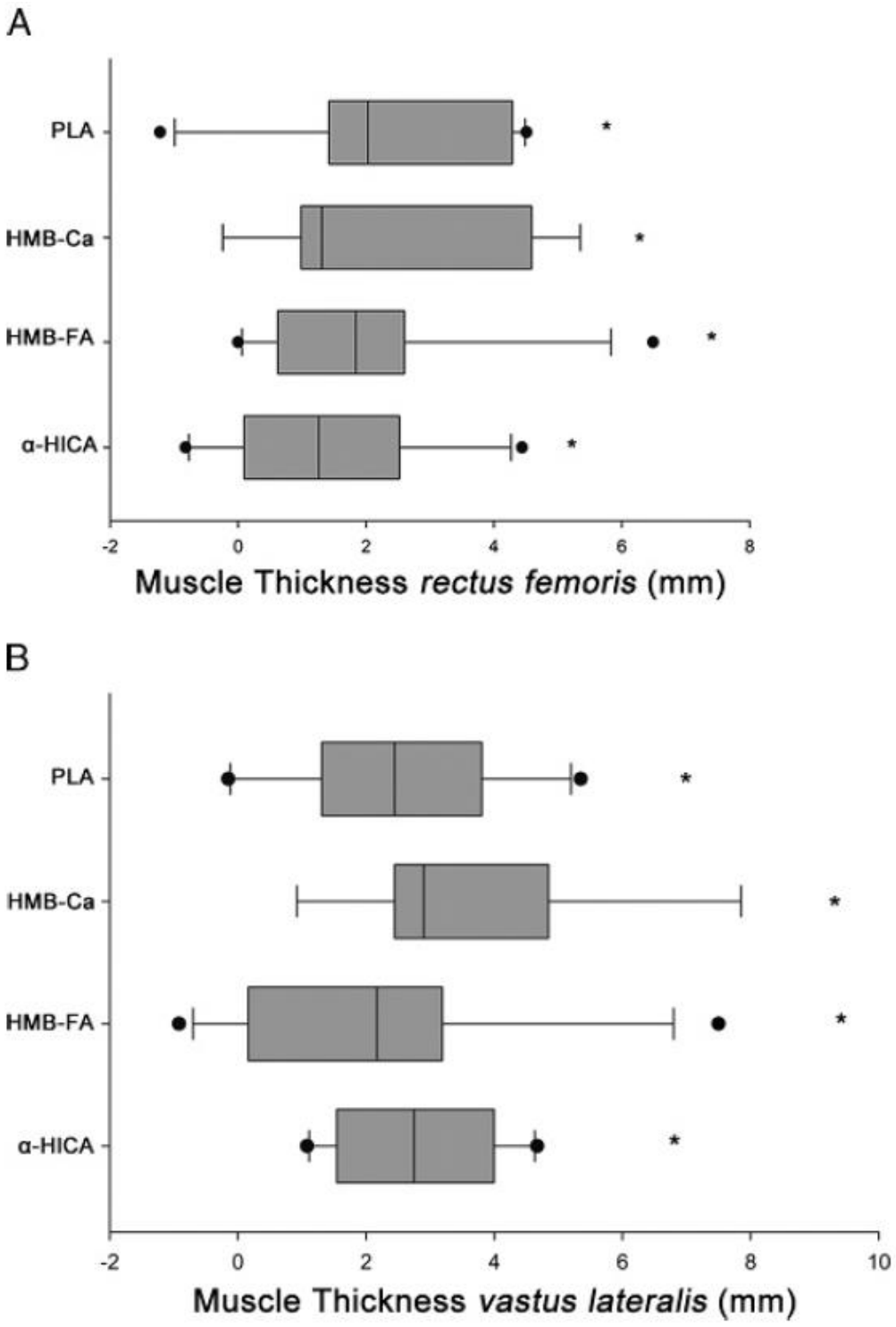

Figure 15. Changes in MT during the 8 week training protocol. Panel A, Δ baseline-week 8 for MT (RF); Panel B, Δ baseline-week 8 MT (VL). Data are shown as box and whisker plots were whiskers are the maximum and minimum and the box represents the interquartile range, the line the group median. Dots represent outliers. *Significantly different ($P < 0.05$) from baseline .

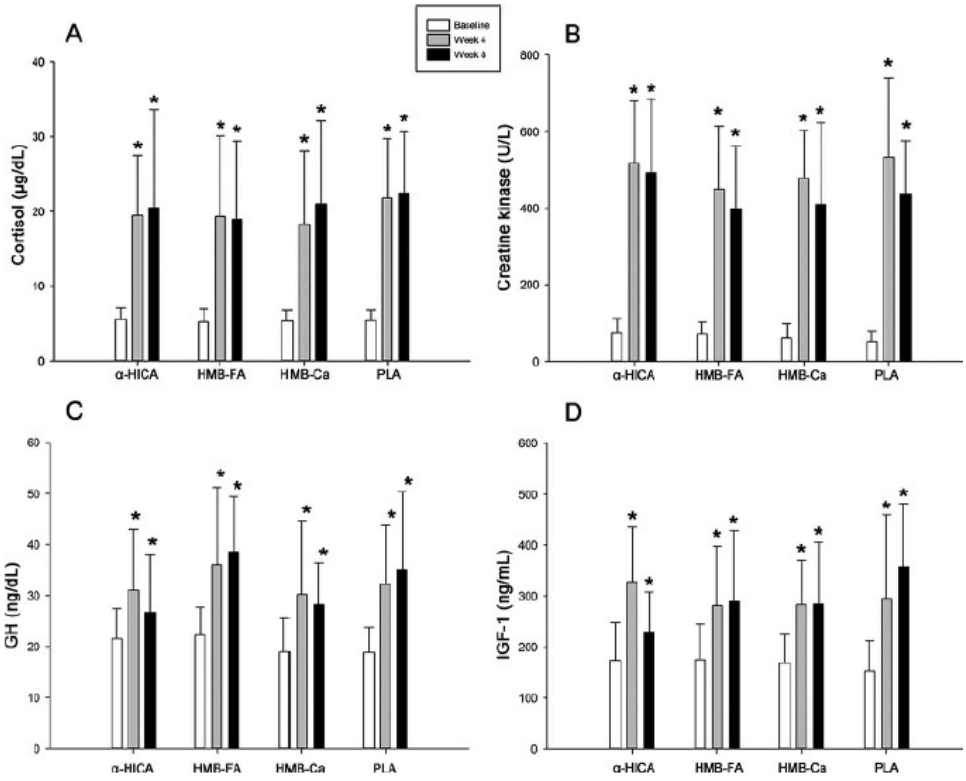

Figure 16. Serum hormone concentrations and creatine kinase activity during the protocol. (A) Cortisol concentration; (B) CK activity; (C) GH concentration; and (D) IGF-1 concentration. *Significantly different ($P < 0.05$) from baseline.

4.4 Discussion

Our study is the first to directly compare the efficacy off-the-shelf commercially available forms of the leucine metabolites α-HICA, HMB-Ca, HMB-FA versus a placebo on resistance training-induced adaptations in performance and muscle thickness in young men consuming a balanced diet with sufficient protein intake. No differences were observed between supplements and placebo in anthropometric measures, muscle thickness, blood markers, or muscle strength and power. We observed training-induced effects for all performance-related variables, including muscle thickness, that was comparable to investigations of similar duration and with a similar group of trainees (10, 11, 28, 29).

Our results of a lack of effect of HMB-Ca are in broad agreement with a variety of other investigations in which HMB-Ca supplementation did not influence resistance training-induced outcomes (11, 29-31). We lack robust data for a comparison of an effect

of α-HICA (3); however, our data provide no support for the concept that α-HICA is anabolic over and above normal protein and energy intake. Limited data exist on HMB-FA, but our data are in direct and sharp contrast to the few studies that have used this supplement (15, 16).

Table 8. Power and strength measures throughout the protocol.

		Baseline	4 wk	8 wk
CMJ height (cm)	α-HICA	39 ± 4	39 ± 4	40 ± 4*
	HMB-FA	37 ± 6	37 ± 8	38 ± 7*
	HMB-Ca	37 ± 7	38 ± 7	39 ± 7*
	PLA	38 ± 7	39 ± 6	39 ± 6*
CMJ power (W)	α-HICA	988 ± 141	995 ± 144	997 ± 148*
	HMB-FA	1009 ± 200	1009 ± 192	1019 ± 203*
	HMB-Ca	1033 ± 219	1048 ± 189	1065 ± 200*
	PLA	1001 ± 123	1026 ± 120	1026 ± 123*
1RM Bench press (kg)	α-HICA	98 ± 29	103 ± 24*	107 ± 24**
	HMB-FA	88 ± 29	96 ± 29*	101 ± 31**
	HMB-Ca	89 ± 17	96 ± 19*	98 ± 16**
	PLA	93 ± 24	101 ± 23*	105 ± 26**
1RM Bench press/BW (kg)	α-HICA	1.3 ± 0.4	1.4 ± 04	1.5 ± 0.4
	HMB-FA	1.1 ± 0.3	1.2 ± 0.3	1.3 ± 0.3
	HMB-Ca	1.1 ± 0.2	1.2 ± 0.2	1.2 ± 0.2
	PLA	1.2 ± 0.2	1.3 ± 0.2	1.4 ± 0.2
1RM Back squat (kg)	A-HICA	124 ± 27	136 ± 29*	145 ± 32**
	HMB-FA	127 ± 31	139 ± 28*	154 ± 33**
	HMB-Ca	128 ± 26	136 ± 26*	141 ± 26**
	PLA	131 ± 22	148 ± 23*	156 ± 25**
1RM Back squat/BW (kg)	α-HICA	1.7 ± 0.4	1.9 ± 0.4	2.0 ± 0.5
	HMB-FA	1.7 ± 0.4	1.8 ± 0.3	2.0 ± 0.4
	HMB-Ca	1.6 ± 0.3	1.7 ± 0.3	1.8 ± 0.4
	PLA	1.7 ± 0.3	1.9 ± 0.3	2.1 ± 0.3

Values are means±SD.
*Significantly different ($P < 0.05$) from baseline.
**Significantly ($P < 0.05$) different from baseline and week 4.

We failed to reproduce the results of Kraemer et al. (24) in recreationally active participants who reported an increase in FFM in young men taking the same dose of HMB-Ca of ~9 kg in 12wk as well as substantially greater increases in CMJ power and 1RM for squat and bench press. However, the supplement used by Kraemer et al. included other dietary ingredients (glutamine, arginine and taurine) which would preclude direct attribution of any changes seen specifically to HMB-Ca; however, we can find no compelling evidence why these other metabolites would be anabolic (32, 33). We also note that there are no meaningful differences in MPS when leucine and HMB in either

calcium or free acid form are compared (14, 34). An obvious difference between our work and that of Kraemer et al. (24) is the training program; however, recent evidence (35) shows that when leucine and HMB-Ca were compared, using the identical training program as that of Kraemer et al., that there were no differences between groups. Moreover, in this work (35) gains in FFM were in-line with recent systematic reviews (25). Meta-analyses show that HMB-Ca modestly augments training-induced increases in FFM in untrained participants and to some extent in elderly populations (6, 19) or older persons in bed rest (5). Regarding HMB-FA, our findings are in stark contrast with previously reported results by Wilson et al. (16) and Lowery et al.(15). However, these studies have been subject to considerable scientific scrutiny and have inherent methodological limitations (17).

Insofar as protein intake is concerned, our study has displayed a mean intake 3.1 ± 0.5 g protein per kg/body weight.day^{-1} which we believe is the highest intake reported with leucine metabolites to date. Other previous studies with HMB-Ca, which are in broad agreement with our results, reported intakes ranging from 1.9 to 2.4 g protein per kg/body weight.day^{-1} (29, 36, 37). These amounts are deemed sufficient according to the latest body of evidence (25). Our higher protein intake is the result of the dietary intervention correcting intakes below 1.6 g protein per kg/body weight.day^{-1} to 2.2 g protein per kg/body weight.day^{1} as suggested by Morton et al. (25). Studies reporting extraordinary body composition outcomes with HMB, have failed to provide absolute energy and protein intakes (15, 16, 24). Thus, whether they were consuming sufficient protein and in an estimated positive energy balance cannot be determined, which poses a serious limitation.

As far as α-HICA is concerned, our findings are in contrast to the FFM increase reported by Mero et al. (3). The participants in this investigation were younger soccer players with no experience in resistance training. Whether α-HICA can improve training-induced changes in body composition or recovery in different populations requires further clarification. Nonetheless, our data provide no support for the contention that α-HICA is anabolic.

Changes in blood markers did not reveal any effect of the leucine metabolites or training protocol on total testosterone. These results do not align with those of some

previous investigations (24, 38) and are in agreement with others (15, 19). The current body of evidence, in addition to our findings, does not support the concept that HMB can increase testosterone levels. As would be expected, training raised both CK, cortisol, IGF-1 and GH. Unlike previous investigations, our findings did not support any reduction in CK activity (2). While circulating CK is a weak proxy marker of muscle damage, it is often used (39). Based on our results, we did not see that either form of HMB or α-HICA was able to prevent exercise induced muscle damage when compared to placebo. It has been questioned, however, whether compounds that are proposed to suppress damage and proteolysis would be effective treatment tools as protein removal is needed to promote clearance of damaged proteins (40).

Cortisol was also elevated during our training protocol, with no effect of any supplement when compared to placebo. Supplementation with HMB has been touted to suppress cortisol elevation to resistance training (16, 24), while others failed to detect significant differences (6, 38). We observed a small but significant rise in GH over time, but with no effect of any supplement. Some reported elevations for GH with HMB (24, 41) while others reported no change with similar protocols (6). In our study we did not detect a change in IGF-1 with any supplement which is in line with some studies (42) but not with others (41, 43). Although IGF-1, GH and testosterone are commonly described as anabolic hormones, current evidence suggests that acute elevations of these hormones does not correlate with resistance training-induced changes in FFM, or strength (44).

None of the leucine metabolites we studied augmented training-induced changes in any index of performance or muscle thickness. Our participants were, according to diet records, in a positive energy balance and consuming a high protein diet. Our results are, at least from a mechanistic perspective, not surprising. It is known that leucine and HMB-FA exert similar effects on protein turnover in humans (14). In addition, there is no apparent anabolic advantage of HMB-FA over HMB-Ca in terms of stimulating protein synthesis (34), despite an apparent difference in bioavailability (34). An important question is how much more efficacious a metabolite of leucine could be than leucine itself in stimulating anabolism and/or suppressing catabolism to affect hypertrophy? We propose that it is unlikely that a metabolite of the same compound would be that much more effective that it could account for the type of growth many have reported (15, 16). This bears particular consideration as leucine and its metabolites share the same canonical

signaling mechanisms leading to stimulation of anabolism and suppression of catabolism (14).

Some of the strengths of the following pragmatic trial are the applicability of this research. While most research uses supplements directly supplied from manufacturers, this was not the case in our trial since all supplements were obtained directly from a supplement store, with no interference from manufacturers. Here we present a tightly controlled pragmatic randomized, double-blinded controlled trial where three commercially available supplements were directly evaluated and compared to placebo. We propose that our findings present a relevant contribution to the current body of evidence regarding these compounds and that our results are noteworthy.

Some weaknesses of our trial include the fact of supplements were presented in capsules and tablets that did not allow for a truly equivalent placebo form. Another limitation is related with the fact that α-HICA was administered in a different fashion than previous research by Mero et al. (3). However, it is unlikely that timing of ingestion of the α-HICA might have influenced results, since different timing protocols have been used with leucine derivatives and no differences were reported (11, 28).

We conclude that when consuming a high protein diet and in an estimated positive energy balance, none of the investigated leucine metabolites resulted in an ergogenic effect on any outcome variable, in young moderately trained men. Our trial represents a true pragmatic trial with supplements that were commercially available as they would be to consumers. Our findings do not support the use of leucine metabolites as supplementation strategy to augment training-induced gains in performance or body composition in young men.

Acknowledgments

The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation. All supplements were freely donated by Body Temple, Lda.

Conflict of interest

FJT, withholds a position as technical manager for Body Temple, Lda a company that sells HMB-Ca and HMB-FA.

The results of the present study do not constitute endorsement by the American College of Sports Medicine.

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CHAPTER 5

No Effect Of HMB Or α-HICA Supplementation On Training-induced Changes In Body Composition[2]

[2]Teixeira FJ, Matias CN, Monteiro CP, Valamatos MJ, Reis JF, Batista A, Oliveira AC, Alves F, Sardinha LB, Phillips SM. No Effect of HMB or α-HICA Supplementation on Training-induced Changes in Body Composition. *European Journal of Sport Science. 2018;1-9. doi:10.1080/17461391.2018.1552723*

No Effect Of HMB or α-HICA Supplementation on Training-Induced Changes In Body Composition

Filipe J. Teixeira, Catarina N. Matias, Cristina P. Monteiro, Maria J. Valamatos, Joana F. Reis, Ana Batista, Ana C. Oliveira, Francisco Alves, Luís B. Sardinha and Stuart M. Phillips

Abstract

β-hydroxy-β-methylbutyrate (calcium: HMB-Ca and free acid: HMB-FA) and α-hydroxyisocaproic acid (α-HICA) are leucine metabolites that have been proposed to improve body composition and strength when combined with resistance exercise training (RET). In this double-blind randomized controlled pragmatic trial, we evaluated the effects of off-the-shelf supplements: α-HICA, HMB-FA and HMB-Ca, on RET-induced changes in body composition, and performance. Forty men were block randomized to receive α-HICA (n=10, Fat-free mass [FFM]=62.0 ± 7.1 kg), HMB-FA (n=11, FFM=62.7 ± 10.5 kg), HMB-Ca (n=9, FFM=65.6 ± 10.1 kg), or placebo (PLA; n=10, FFM=64.2 ± 5.7 kg). The training protocol consisted of a whole-body resistance training routine, thrice weekly for 8 weeks. Body composition was assessed by dual-energy x-ray absorptiometry (DXA) and total body water (TBW) by whole body bioimpedance spectroscopy (BIS), both at baseline and at the end of weeks 4 and 8. Time-dependent changes were observed for increases in trunk FFM ($p < 0.05$). No statistically significant between-group or group-by-time interactions were observed. Supplementation with HMB (FA and Ca) or α-HICA failed to enhance body composition to a greater extent than placebo. We do not recommend these leucine metabolites for improving body composition changes with RET in young adult resistance trained men.

Key Words: *resistance training, fat-free mass, leucine metabolites*

5.1 Introduction

Leucine is the primary amino acid agonist, as well as a substrate, for stimulating muscle protein synthesis (1-3) and is a key driver of anabolism (4). It is therefore unsurprising that metabolites of leucine such as β-hydroxy-β-methylbutyrate (HMB) (5, 6) and α-hydroxyisocaproic acid (α-HICA) (7) have been suggested to increase fat-bone-free (i.e., lean) body mass and improve performance. Research on HMB (a deaminated and decarboxylated metabolite of leucine) (8) and its effects on fat-free mass increases in (FFM) and performance enhancement with resistance exercise training (RET) is extensive, but evidence-based analysis of these effects shows trivial enhancement (5) or no positive impact on either RET-induced body composition or performance (6). The deaminated form of leucine, α-HICA, is a far less well studied compound, with only two investigations being performed in humans (7, 9) and a case study in a type I diabetic patient (10).

There are reports of considerable RET-induced gains in muscle mass and concurrent reductions in fat mass (FM) with resistance exercise training (RET) and HMB supplementation (11-13). However, several of these investigations (12, 13) have been challenged on study design and the plausibility of the upper limits of muscle growth (14, 15). Despite equivocal evidence-based reviews on the effectiveness of HMB in promotion of RET-induced gains in FFM and performance (5, 6, 8) in both novice and experienced athletes, a recent update and recommendation placed HMB in a category of supplements for which there was strong evidence to support efficacy and apparently safe (16). While the safety of HMB is established (5, 6, 8) its efficacy is questionable, at least based on results of several meta-analyses (5, 6, 8).

We have previously reported that RET-induced gains in muscle thickness, a valid proxy of hypertrophy, and performance, were not different between placebo or HMB-Ca (calcium salt) and FA (free acid) or α-HICA-supplemented groups in an 8 weeks RET study (9). Following up on our previous investigation (9) we report here further results that add to our understanding of the impact, or lack thereof, of the leucine metabolites HMB and α-HICA in body composition. Since more than six weeks of supplementation has been proposed as a limit to obtain positive benefits with HMB (17), our trial was 8 weeks in duration. Based on previous reports (11-13), our hypothesis was that

participants, in an estimated positive energy balance consuming sufficient protein, would benefit from supplementation with leucine metabolites – HMB-FA, α-HICA and HMB-Ca to improve body composition during a program of RET.

5.2 Methods

ETHICS

All procedures were approved by the Faculty of Human Kinetics Institutional Review Board (approval number 15/2017), following all the guidelines regarding human research set out in the declaration of Helsinki. The trial was registered at clinicaltrials.gov as NCT03511092. Prior to engaging in any of the study procedures, the purpose and design of the study, the data collection methodologies and all potential risks and benefits were explained to potential research participants. All selected participants gave their verbal and written informed consent before enrolling.

GENERAL STUDY DESIGN

As previously reported (9), fifty-three men were initially recruited according to the eligibility criteria, with forty completing the investigation. Participants were between the ages of 18 and 45 y and were recruited from social networks and local gyms. For further details, please refer to our previous work (9).

Supplements were freely donated from shelf-stock from a local supplement store and although brands presented a certificate of analysis (COA), supplements were additionally analyzed by a third party certified and accredited laboratory to confirm compliance with the brand's package COA. All supplements presented similar concentrations with the brand's COA, as previously reported (9). An inert and safe substance was used as placebo (magnesium stearate).

Assignment of subjects to groups was performed according to a randomly generated list and blocked in varying block sizes with participants matched for baseline grip strength, age and DXA-measured FFM. No baseline statistically significant

differences existed among groups for (handgrip strength, age, FM or FFM as previously described (9) (Table 9).

Table 9. Baseline characteristics of the participants.

	Age (years)	Height (cm)	Weight (kg)	FFM (kg)	Handgrip dominant side (kg)
α-HICA	31 ± 8	172 ± 7	73.3 ± 9.6	62.0 ± 7.1	50.4 ± 12.4
HMB-FA	30 ± 8	174 ± 5	77.0 ± 13.7	62.7 ± 10.5	51.9 ± 8.1
HMB-Ca	34 ± 4	177 ± 6	78.2 ± 10.6	65.6 ± 10.1	55.5 ± 5.6
PLA	31 ± 7	174 ± 6	75.8 ± 6.9	64.2 ± 5.7	50.9 ± 9.7

Values are means ± SD.

The training protocol consisted on whole body hypertrophy-type resistance training routine for intermediate-trained individuals and consisted of 3 training sessions per week for 8 weeks (Table 10). Evaluations of strength, body composition and performance took place at baseline and weeks 4 and 8.

Table 10. Characteristics of the training protocol during the study.

Weeks	Sets	Reps	Rest
1 and 2	3	12 RM	60 s
3	4	12 RM	60 s
4	3	10 RM	90 s
5 and 6	4	10 RM	90 s
7 and 8	4	8 RM	120 s

PARTICIPANTS

The sample was comprised of healthy men between 18 and 45 years old, currently engaged in resistance training for at least 1 year and at least 3 times per week. Sample size was calculated from previous investigations aiming to demonstrate similar adaptive responses between conditions, with inclusion and exclusion criteria as previously reported (9).

BODY COMPOSITION

Body composition was determined at baseline, week 4 and 8 by dual-energy x-ray absorptiometry (DXA) and bioimpedance spectroscopy (BIS). Participants underwent a DXA scan according to the procedures recommended by the manufacturer on a Hologic Explorer-W, fan-beam densitometer (Hologic, Waltham, Massachusetts, USA) as described by our group previously (9). Analyses provided total, fat-free and fat masses from whole body and different subregions.

Although software automatically generates regions (i.e. arms, legs and trunk), those were adjusted manually by the technician. Specifically, trunk region was separated from the legs by a horizontal line right above the iliac crest (lower boundary) and from the head by neck cut (upper boundary) (18). The trunk region includes the neck, chest, abdominal and pelvic areas except the gluteal area that was included into legs (19). The coefficients of variation in our laboratory, based on 10 young active adults (five males and five females), is 1.6% for BMC, 1.7% for FM, and 0.8% for FFM (20).

Total body water (TBW) and both the extracellular and intracellular water were determined by whole body resistance and reactance using BIS model 4200 (Xitron Technologies, San Diego, CA, USA) after a 10-minute rest period in a supine position. Four electrodes were placed on the dorsal surfaces of the right hand and foot (21). The software performed biophysical modelling according to the impedance data. Extracellular water and intracellular water were calculated individually. Total body water was calculated as the sum of extracellular and intracellular water.

SUPPLEMENTATION AND DIET CONTROL

Each participant received a supplement containing either α-HICA (HICA, Onsalesit, SA, Funchal, Portugal), HMB-FA (Beta-TOR, Body Attack, Hamburg, Germany), HMB-Ca (HMB Mega Caps 1250, Olimp Labs, Pustynia, Poland), or placebo (magnesium stearate, EightJuice, Seixal, Portugal). Detailed procedures regarding double-blinding, samples and randomization have been already described by our group previously (9). Participants' ingested supplements or placebo thrice daily, alongside with meals or prior to training, according with previous research: 3 x 500 mg for α-HICA (7) and 3 x 1 g for HMB-FA (13), HMB-Ca (11) or placebo.

Dietary counselling was provided by trained dieticians to assure sufficient energy and protein in order to allow for training-induced gains of lean mass. Participants self-reported three-day dietary intake through dietary logs at the baseline and at the end of week 4 and 8. Before the beginning of the study, if participants were not ingesting at least 1.6 g of protein per kg/body weight.day^{-1} (22) and at least 45 kcal per kg of FFM (23), a registered dietitian would provide counselling on foods to consume in order to adjust the protein and/or energy intake. A more detailed description regarding the dietary counselling, diet composition and compliance with supplement intake, is available from our previous work (9).

TRAINING AND EXERCISE PROTOCOLS

The resistance training protocol was designed according to the guidelines for hypertrophy type of resistance training for intermediate-trained individuals and consisted of 3 sessions per week during an 8-week period (70-80% 1RM), with a minimum of 48 hours interval between sessions (24). A detailed description of the training methodology may be consulted according to our previous work (9) with further training protocol details being provided in table 10.

STATISTICS

Sample size was calculated through an a priori power analysis (G*Power Version 3.1.9.2, Heinrich Heine Universitat Dusseldorf, Germany), based on FFM changes from previous investigations (12) and power of 0.80 and alpha of 0.05. Statistical analysis was performed using IBM SPSS statistics version 22.0 (IBM, Chicago, Illinois, USA). Normality of the distribution of variables was tested by Shapiro-Wilk test. Between groups baseline characteristics and Δ baseline-week 8 assessments, were analyzed by a 1-way analysis of variance (ANOVA), since normality was observed. Time and time-by-group interactions were evaluated by repeated-measures ANOVA. Overall significance level for α was set at $p \leq 0.05$.

5.3 Results

No differences ($p > 0.05$) were found for dietary intake throughout the study. As previously described, high compliance levels were reported among participants regarding both supplement intake and training sessions (9).

No differences between groups were observed ($p > 0.05$) for body weight or TBW from baseline to week 4 and 8. No time or time by group interactions were found ($p > 0.05$) for whole-body FFM (Δ: α-HICA -0.1 ± 1.0 kg; HMB-FA 0.1 ± 1.5 kg; HMB-Ca 0.6 ± 2.0 kg; PLA 0.6 ± 0.8 kg), and FM (Δ: α-HICA -0.1 ± 1.0 kg; HMB-FA -0.2 ± 1.4 kg; HMB-Ca 0.0 ± 1.6 kg; PLA: -0.3 ± 0.9 kg) (figure 20).

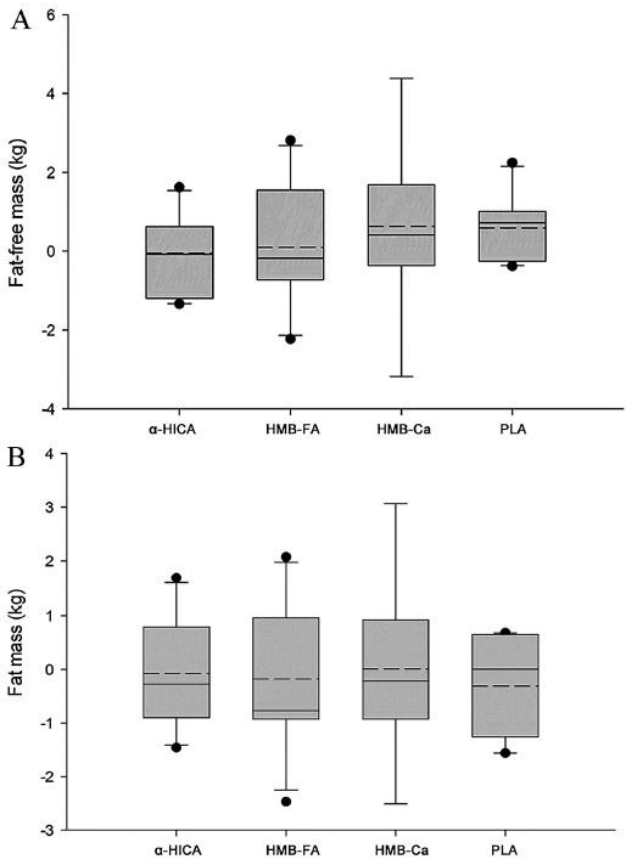

Figure 17. Changes in whole-body FFM and FM during the 8-week training protocol. Panel A: Δ Baseline-week 8 for FFM; Panel B: Δ Baseline-week 8 for FM. Data are shown as box and whisker plots where whiskers are the maximum and minimum and the box represents the interquartile range, the line the group median and the dashed line the group mean. Dots represent high and low responders. *Different from baseline ($p < 0.05$)

Additional regional body composition analysis showed a time effect ($p < 0.05$) from baseline to week 8 for trunk FFM only (Δ: α-HICA 0.2 ± 0.6 kg; HMB-FA 0.2 ± 0.8 kg; HMB-Ca 0.8 ± 1.2 kg; PLA: 0.5 ± 0.7 kg, $p < 0.001$) (figure 21), with no further effect on legs, arms or appendicular FM or FFM being found. No differences between groups ($p > 0.05$) were found for any whole-body or regional body composition outcome variable.

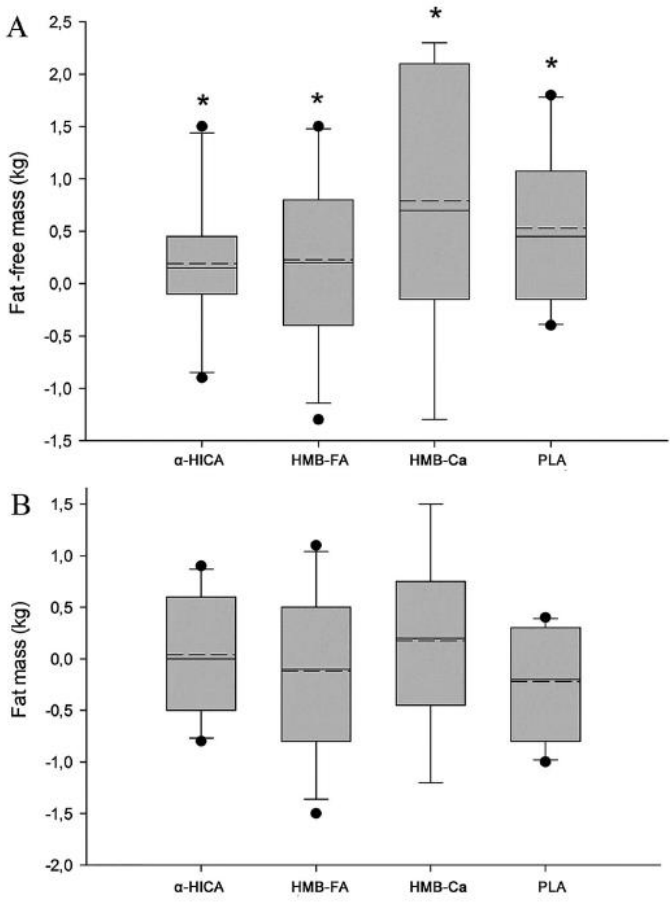

Figure 18. Changes in trunk FFM and FM during the 8-week training protocol. Panel A: Δ Baseline-week 8 for trunk FFM; Panel B: Δ Baseline-week 8 for trunk FM. Data are shown as box and whisker plots where whiskers are the maximum and minimum and the box represents the interquartile range, the line the group median and the dashed line the group mean. Dots represent high and low responders. *Different from baseline ($p < 0.05$)

5.4 Discussion

This is the first, placebo-controlled research study, that directly compared several leucine metabolites (HMB-Ca, HMB-FA, and α-HICA) and their effects on RET-induced

changes in body composition. No differences were found between groups with either supplement or placebo for RRT-induced increases in FFM or decreases in FM, with only a small increase in trunk FFM being reported, due to training. We propose that our results are a valuable contribution to this field and further align with other recent findings (25) and are in broad agreement with previous reviews (5, 6) showing a lack of marked effect of HMB on hypertrophy. Our results do, however, differ from previously reported extraordinary results (11-13) which have led more recent reviews to recommend HMB as an effective strategy to enhance FFM gains (16, 26).

Kraemer et al. (2009) observed that recreationally active individuals supplemented with HMB-Ca gained ~9.3 kg FFM and decreased their FM by ~4.9% over 12 weeks. Wilson et al. (2014), in trained men reported gains of 7.4 kg FFM, over 12 weeks, and a FM reduction of 5.4 kg. In a study from the same subjects, Lowery al. (2016), reported FFM gains, over the same time period, of 8.5 kg and a FM reduction of 8.5%. These body composition results are surprising and are of a similar magnitude to those seen with RET and weekly administration of 300 mg of testosterone enanthate (27) in terms of FFM gains. Regarding FM, we note that the reductions seen in these investigations (11-13) exceed those induced with orlistat or sibutramine in active duty soldiers and obese individuals, respectively (28, 29).

Some limitations should be pointed out regarding previous research investigations (11-13). First, none reported absolute values for energy or macronutrient intake and neither Wilson et al. (2014) nor Lowery et al. (2016) appeared to have controlled for variations in TBW, which may confound DXA assessments (30). The lack of absolute values for dietary intake (energy and macronutrients), does not allow us to assess whether participants in these investigations (11-13) were in an estimated positive energy balance or consuming sufficient protein. It has been shown, that free leucine supplementation (3.0 g/day) fails to increase muscle mass and strength when sufficient protein and energy are provided (31), thus supplementing with these derivatives, under different dietary contexts could lead to different outcomes.

We have previously shown, in the same group of trainees, that when consuming sufficient protein and energy, we saw no advantage with any leucine metabolite with respect to increases in skeletal muscle thickness (9). Similarly, Jakubowski et al. (25)

reported no differences when the same amount of either leucine or HMB-Ca were consumed, over 12 weeks, using the same training protocol reported to be used in previous works (11-13). These results are not surprising, at least from a mechanistic perspective, since leucine metabolites share the same biochemical signaling pathways as leucine and previous research showed no difference between leucine and HMB insofar as stimulation of muscle protein synthesis (MPS) is concerned (32).

It has been suggested that a possible action for HMB to reduce BF may be through Sirt1 and 3 (Silent information transcripts 1 and 3) and AMPK (adenosine monophosphate kinase) – a mechanism that has also been reported with leucine (13) – however, these mechanisms are derived from in vitro and/or animal investigations (33, 34), while human investigations for 12 weeks, in novice trainees, show no effect of leucine in reducing BF (35). Another argument used to support previously reported extraordinary results (11, 13), is the addition of other ostensibly ergogenic amino acids (arginine, glutamine and taurine) or ATP. However, an extensive literature search reveals no reason why these compounds would be anabolic when sufficient energy and protein are consumed (15, 36, 37).

Insofar as α-HICA is concerned, only two investigations analyzed its efficacy in humans - one in young soccer players (7) and other in a type I diabetes case study (10). Research in young resistance trained participants is therefore lacking, with our results not providing support that α-HICA is anabolic over and above sufficient energy and protein intake in this group of trainees.

Some of the strengths of our investigation are related with its applicability since the supplements were acquired off the shelf and our study was conducted without influence of industrial sponsorship. Additionally, the participants' diets were controlled and sufficient energy and protein intake were assured. No differences regarding dietary intake were detected, which is in our view important, since a glycogen increase from a higher carbohydrate intake might have confounded the body composition results (38, 39). We accounted for variation in TBW throughout the intervention, with no significant changes being detected. Small increases in trunk FFM were observed due to training. These gains are not by surprising, since the majority of the training protocol targeted

muscles in this region (i.e., back and chest), which probably led to a local hypertrophic outcome (40).

We have previously shown that hypertrophy occurred in the quadriceps muscle and that proxy markers of muscle damage increased with this training protocol, our study failed to detect statistically significant gains in whole-body (DXA-measured) FFM, although HMB groups and placebo showed non-significant increases after 8 weeks (9). Considering similar investigations (Jakubowski et al., 2018), we hypothesize that if our trial was extended for four more weeks a training effect would have been reported.

In conclusion, after a whole-body resistance training together with a high protein diet and an estimated positive energy balance, none of either HMB-Ca, HMB-FA, or α-HICA resulted in an ergogenic effect on FM and FFM. Our findings do not support the use of any leucine metabolite to improve body composition or performance in young adult men.

Acknowledgments

FJT made all laboratory evaluations and wrote the manuscript. CNM, CPM, MJV, JFR, assisted on measurements and development of the manuscript. AB and ACO assisted on measurements. FA, LBS and SMP reviewed and assisted on the manuscript development. All authors approved the final version of the manuscript. All supplements were freely donated by Body Temple, Lda.

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Conflict of interest

At the time of data collection FJT, withheld a position as technical manager for Body Temple, Lda a company that sells HMB-Ca and HMB-FA.

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CHAPTER 6

Leucine Metabolites Do Not Attenuate Training Induced Inflammation in Young Resistance Trained Men[3]

[3]Teixeira FJ, Matias CN, Monteiro CP, Valamatos MJ, Reis JF, Morton RW, Alves F, Sardinha LB, Phillips SM. Leucine Metabolites Do Not Enhance Training-induced Inflammation in Young Resistance Trained Men. *Journal of Sport Sciences*. (Under Review)

Leucine Metabolites Do Not Attenuate Training Induced Inflammation In Young Resistance Trained Men

Filipe J. Teixeira, Catarina N. Matias, Cristina P. Monteiro, Maria J. Valamatos, Joana F. Reis, Robert W. Morton, F. Alves, Francisco, Luís B. Sardinha and Stuart M. Phillips

Abstract

Some leucine-derived metabolites have been proposed to reduce training-induced inflammation after exercise, however, there is scant evidence for this assertion. We conducted a double-blind randomized controlled pragmatic trial where 40 male participants were allocated into 4 groups: α-hydroxyisocaproic acid group ([α-HICA], n=10, Fat-free mass [FFM]=62.0 ± 7.1 kg), β-hydroxy-β-methylbutyrate free acid group ([HMB-FA], n=11, FFM=62.7 ± 10.5 kg), calcium β-hydroxy-β-methylbutyrate group ([HMB-Ca], n=9, FFM=65.6 ± 10.1 kg), or placebo group ([PLA]; n=10, FFM=64.2 ± 5.7 kg). An 8-week whole-body resistance training routine (3 training sessions per week) was employed to induce gains in skeletal-muscle thickness. Skeletal muscle thickness (MT), one repetition maximum (1RM), interleukin-6 (IL-6), high-sensitivity C-reactive protein (hsCRP) and tumor necrosis factor alpha (TNF-α) were assessed at baseline and at the end of weeks 4 and 8. Time dependent changes were detected from baseline to week 8 for MT (vastus lateralis: $p = 0.009$; rectus femoris: $p = 0.018$), 1RM (back squat and bench press: $p < 0.001$), IL-6, hsCRP (both $p < 0.001$) and TNF-α ($p = 0.045$). No differences were found between groups at any time point such that no leucine metabolite attenuated inflammation during training. In addition, using backwards elimination regressions, no circulating inflammatory marker consistently shared variance with the change in any outcome (MT or 1RM). Lastly, changes in circulating inflammatory markers were not correlated with changes in muscle hypertrophy or strength in young, resistance trained individuals.

***Key words**: inflammation; leucine metabolites; hypertrophy; strength*

6.1 Introduction

Inflammation is a hallmark of several muscle wasting conditions (1). In disease states, skeletal muscle loss is associated with increased production of proinflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6), and acute phase protein release (2), which may result in an increase in resting energy expenditure and augment muscle protein breakdown (MPB) (3).

It should however be noted, that muscle-derived IL-6 acts in a different fashion than macrophage or adipocyte released IL-6, since it serves to augment hepatic glucose and adipose tissue fatty acid release to provide sufficient fuel to meet the extra metabolic demand from exercise and also exerts local anti-inflammatory action through the inhibition of TNF-α and interleukin 1 β (IL-1β) release (4). Chronic inflammation might negatively impair skeletal muscle homeostasis, especially when linked to disease states, inflammation is deemed vital for muscle adaptations from resistance exercise (RE), further promoting skeletal muscle increases (5).

Leucine metabolites β-hydroxy methylbutyrate (HMB), in both the calcium salt form (HMB-Ca) and free acid form (HMB-FA), have been proposed to prevent exercise induced increases in inflammatory markers TNF-α, IL-6 and C-reactive protein (CRP) (6-8) while data regarding α-hydroxy isocaproic acid (α-HICA) is currently lacking. It has been suggested that these inflammatory modulation properties attributed to HMB may be the result of decreased muscle damage, direct action upon T-lymphocytes (9) and/or the inhibition of the production of reactive oxygen species (ROS) via TNF-α inhibition (7).

Since the attenuation of IL-6, TNF-α and CRP were reported with formulas containing more ingredients besides HMB (6) and as a result of acute bouts of RE (7) or in combination with cold water immersion (CWI) (8), further research should be conducted to clarify whether these compounds may in fact attenuate inflammatory markers and influence skeletal muscle accretion. Since longer duration trials, in trained subjects, have failed to display any influence of some leucine metabolites regarding these biomarkers (10-12), further research is warranted.

We have previously reported that RE-induced gains in muscle thickness, performance and action upon anabolic/catabolic hormones (insulin-like growth factor 1 [IGF-1], testosterone, human growth hormone [GH] and cortisol) or proxy markers of muscle damage (creatine kinase [CK]) were not different between placebo, HMB-Ca, HMB-FA and α-HICA-supplemented groups, after 8 weeks of a RE protocol (13). Following up on our previous investigation, we report further results regarding IL-6, TNF-α and CRP that may be important for the understanding of the impact of these leucine metabolites regarding exercise induced inflammation. To our knowledge, no investigation has directly compared these leucine metabolites on RE-induced inflammation. Our working hypothesis, based on previous results (6-8), was that young resistance trained participants consuming sufficient protein and in an estimated positive energy balance would benefit from these supplements by their ability to attenuate inflammation.

6.2 Methods

ETHICS

This double-blind, randomized, placebo-controlled trial received ethics approval from the Faculty of Human Kinetics' ethics committee (approval number 15/2017) and conformed to all standards of human research set out in the declaration of Helsinki. The trial was further registered at clinicaltrials.org as NCT03511092. Before engaging in any experimental procedure, a verbal and written explanation of the study procedures was provided to all participants before enrolling and they signed an informed consent.

STUDY DESIGN

Participants were recruited according to previously described eligibility criteria (13) from local gyms and social networks. As previously reported, supplements were donated from shelf-stock from a local supplement store and subject to a third-party analysis to confirm content compliance (13). All supplements, according to the third-party analysis, contained similar concentrations of either HMB-Ca, HMB-FA and α-HICA (13). Magnesium stearate was used as a placebo due to its lack of biological activity and safety. The RE protocol was a whole-body hypertrophy-type resistance training

routine that was comprised of 3 training sessions per week. The supplementation and training protocol were sustained throughout the entire study duration (8 weeks), with muscle thickness (MT), strength measurements and inflammatory markers being assessed at baseline and end of week 4 and 8. For a more detailed view regarding the study design please refer to figure 22.

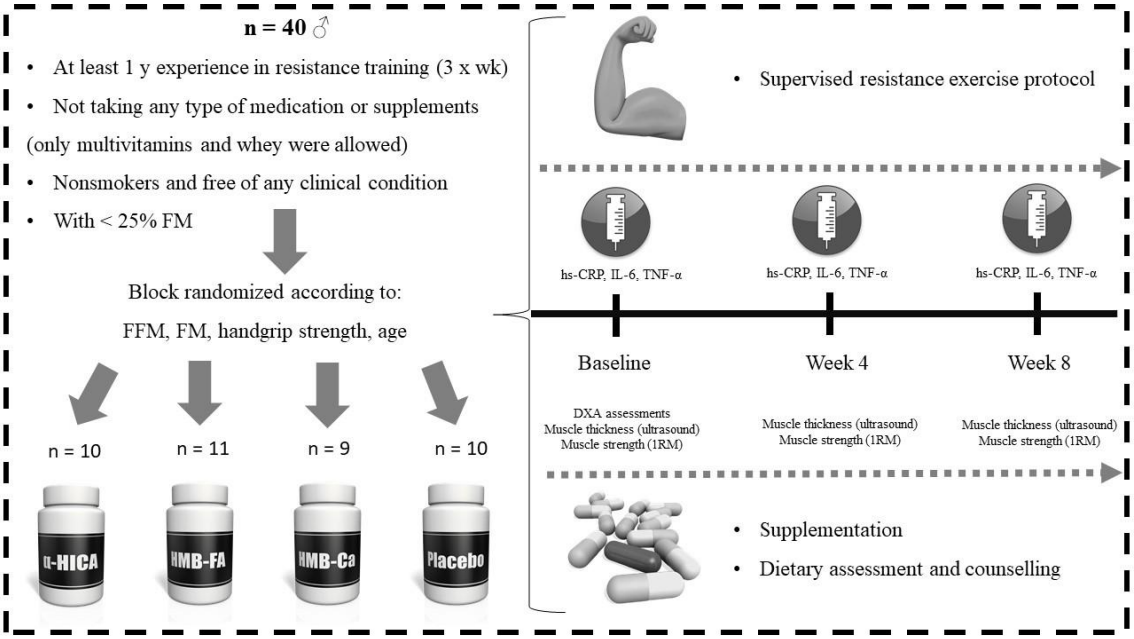

Figure 19. Study design.

SAMPLE SELECTION

Fifty-three healthy men between 18 and 45 years old, currently engaged in RE for at least 1 year (3 training sessions per week) were recruited. The inclusion and exclusion criteria were applied has previously described (13). Forty subjects completed the investigation with the dropout reasons being mentioned in our previous work (13).

BODY COMPOSITION

Dual x-ray absorptiometry (DXA) was used to assess baseline body composition. Participants underwent a DXA scan according to the procedures recommended by the manufacturer on a Hologic Explorer-W, fan-beam densitometer (Hologic, Waltham, Massachusetts, USA). Analyses provided whole body fat-free and fat masses.

Muscle thickness (MT) of the vastus lateralis (VL) and rectus femoris (RF) was assessed at rest using B-mode ultrasound imaging with a 9-cm-long 10 MHz linear-array transducer (model EUB-7500, Hitachi Medical Corporation, Tokyo, Japan). Scans were performed according to previous studies (14) from the mid-belly corresponding to 39% (VL) and 56% (RF) of the distance from the proximal edge of the patella to the anterior superior iliac spine.

MUSCLE STRENGTH

Muscle strength was assessed at baseline by one repetition maximum (1RM) of the back squat and bench press. These assessments were repeated at the end of week 4 and 8 (figure 22). The evaluation of 1RM was obtained from the back squat and bench press exercises on a Multipower machine (Model-M953; Technogym, Cesena, Italy). All assessments were conducted according to the National Strength and Conditioning Association (NSCA) and supervised by an NSCA-certified strength and conditioning specialist.

SUPPLEMENTATION AND DIET CONTROL

Randomly generated groups received either α-HICA (HICA, Onsalesit SA, Funchal, Portugal), HMB-FA (Beta-TOR, Body Attack, Hamburg, Germany), HMB-Ca (HMB Mega Caps 1250, Olimp Labs, Pustynia, Poland) or placebo (magnesium stearate, EightJuice, Seixal, Portugal). All procedures (double-blinding, sample distribution and randomization) have already been described in our previous work (13). Supplements were ingested thrice daily: α-HICA (3 x 500 mg) (15), HMB both FA and Ca forms (3 x 1 g) (10, 16) or placebo.

To provide optimal hypertrophic outcomes, trained dieticians provided counselling throughout the study. All participants had to ingest at least 1.6 g of protein per kg/body weight.day^{-1} (17) and a minimum of 45 kcal per kg of FFM.day^{-1} (18). A thoroughly detailed description regarding self-reported dietary intakes, dietary counselling, diet composition and compliance may be consulted in our previous work (13).

TRAINING PROTOCOL

The RE protocol was designed to induce hypertrophy in intermediate-trained

individuals and consisted of 3 weekly sessions, for 8 weeks, between 70 and 80% 1RM (19). A minimum of 48 hours interval between sessions was implemented to assure proper recovery. For a more detailed description regarding the training program, please refer to our previously published work (13).

BLOOD MARKERS

Blood samples were collected at baseline and at the end of week 4 and 8 into ethylenediaminetetraacetic acid (EDTA) tubes, then centrifuged at 500 *g* at 4 ºC for 15 min, with plasma being thawed at -80 ºC. Plasma was further analysed for IL-6, high-sensitivity C-reactive protein (hsCRP) and TNF-α concentrations at the core Laboratory of McMaster University Medical Centre. IL-6 and TNF-α were analysed using a Bio-Plex reagent Kit and a Bio-Plex reader (Bio-Rad Laboratories, Hercules, CA) by enzyme-linked immunosorbent assay (ELISA), while high-sensitivity C-reactive protein (hsCRP) was analysed using a commercially available high-sensitivity CRP-Latex Kit (Pulse Scientific, Burlington, ON, CA) and an Express Plus autoanalyzer (Chiron Diagnostics Co, Walpole, MA). Intra-assay coefficients of variation reported from our lab are <6%, <4.5% and <3.5%, for IL-6, TNF-α and hsCRP, respectively. Concentrations of these inflammatory markers at weeks 4 and 8 were corrected for plasma volume variation with haemoglobin concentration and haematocrit according to Dill and Costill (20).

STATISTICAL ANALYSIS

Sample size was calculated through an a priori power analysis (G*Power Version 3.1.9.2, Heinrich Heine Universitat Dusseldorf, Germany), based on FFM changes from previous investigations (11) and power of 0.80 and alpha of 0.05. Statistical analysis was performed using IBM SPSS statistics version 22.0 (IBM, Chicago, Illinois, USA). Normality of the distribution of variables was tested by Shapiro-Wilk test. Between groups baseline characteristics and Δ baseline-week 8 assessments were analyzed by a 1-way analysis of variance (ANOVA), since normality was observed. Time and time-by-group interactions were evaluated by repeated-measures ANOVA. Overall significance level for α was set at $p \leq 0.05$.

Additionally, backwards elimination regression equations were generated in effort to elucidate if a combination of inflammatory markers shared variance with any primary outcomes (Δ% combined 1RM, Δ% VL MT, and Δ% RF MT) or baseline values

(combined 1RM, VL MT, and RF MT). The probability of F was used as our stepwise criteria with entry at 0.05 and removal at 0.06. Scatter plots with *ZRESID plotted against *ZPRED were used to assess linearity and heteroscedasticity when one or more independent variables were retained in the model.

6.3 Results

As previously reported (13) muscle strength increased from baseline to week 8 ($p < 0.001$) for 1RM back squat (α-HICA: 18.5% ± 18.9%; HMB-FA: 23.2% ± 16%; HMB-Ca: 10.5% ± 13.8%; PLA: 19.7% ± 9%) and 1RM bench press (α-HICA: 13.8% ± 19.1%; HMB-FA: 15.5% ± 9.3%; HMB-Ca: 10% ± 10.4%; PLA: 14.4% ± 11.3%). Also, MT increased for VL (α-HICA: 2.9 mm ± 1.3 mm; HMB-FA: 2.3 mm ± 2.3 mm; HMB-Ca: 3.7 mm ± 2 mm; PLA: 2.5 mm ± 1.7 mm) and RF (α-HICA: 1.4 mm ± 1.6 mm; HMB-FA: 2 mm ± 1.8 mm; HMB-Ca: 2.3 mm ± 2 mm; PLA: 2.3 mm ± 1.8 mm) from baseline to week 8 (VL: $p = 0.009$; RF: $p = 0.018$).

Inflammatory markers (IL,6, hsCRP and TNF-α) increased from baseline to week 8, with no differences between groups (figure 23). According to backwards elimination regression equations, no circulating inflammatory marker consistently shared variance with the percent change in any outcome (i.e., no independent variable shared a significant amount of variance with multiple outcomes and/or at multiple times of measurement). The coefficients of determination (i.e., R^2) values were low (<0.25) for all outcomes at each time of measurement, indicating that little of the variation seen in any outcome can be explained by any model fitted here (table 11 and 12).

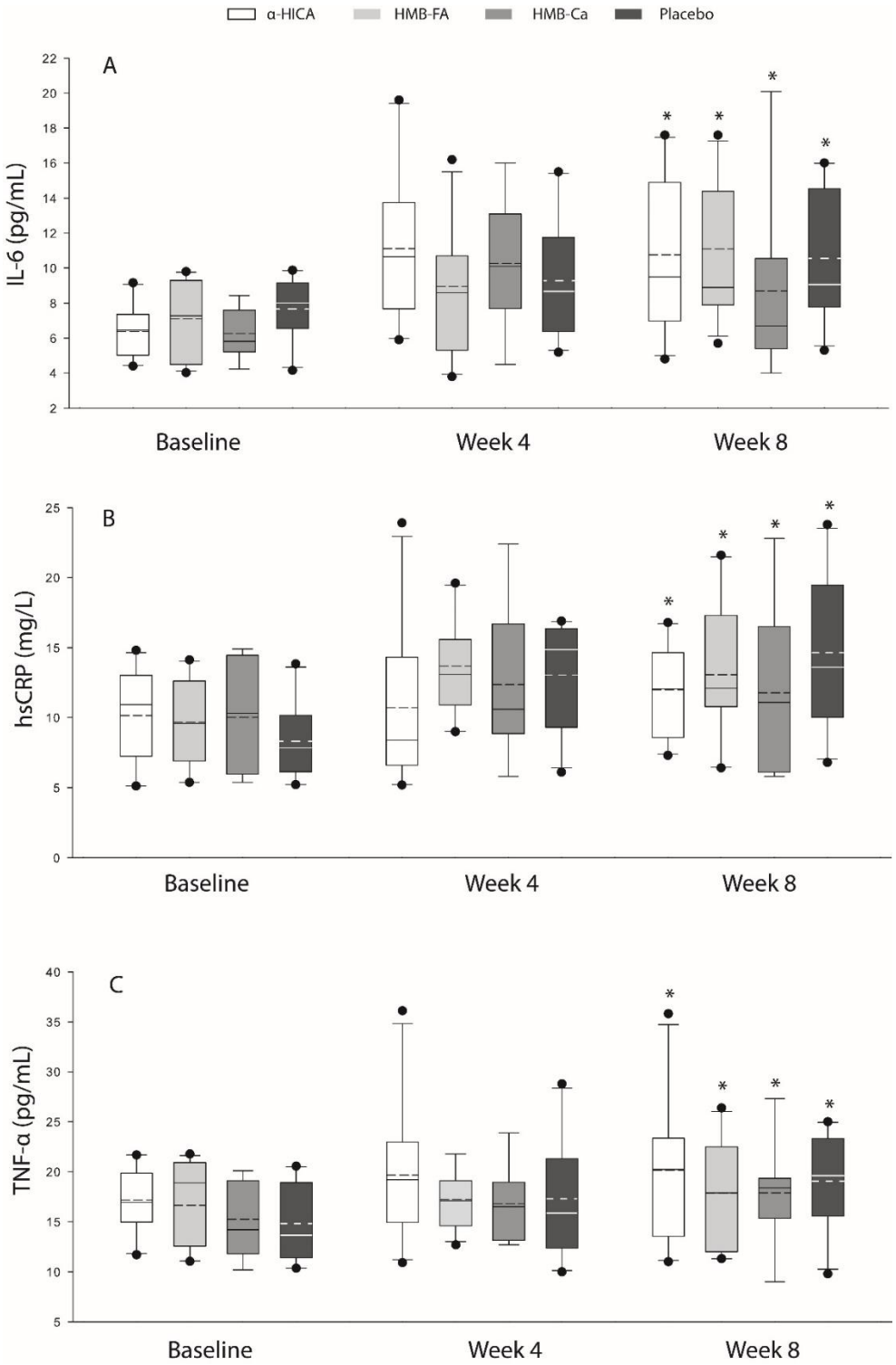

Figure 20. Inflammatory markers assessments during the 8-week training protocol. Panel A: IL-6; Panel B: hsCRP; Panel C: TNF-α. Data are shown as box and whisker plots where whiskers are the maximum and minimum and the box represents the interquartile range, the line the group median and the dashed line the group mean. Dots represent high and low responders. *significantly different from baseline.

Table 11. Backwards elimination regression final output between resting inflammatory markers and % changes in strength and MT.

	Baseline					Week 4					Week 8			
	Estimate	SEM	t-value	p-value		Estimate	SEM	t-value	p-value		Estimate	SEM	t-value	p-value
%△ 1RM (combined)														
Intercept	56.38	9.46	5.96	<0.01	Intercept	32.97	3.30	10.00	<0.01	Intercept	49.46	8.50	5.82	<0.01
hs-CRP	-2.46	0.94	-2.62	0.01						**IL-6**	-1.60	0.76	-2.09	0.04
	F = 6.85	*df = 39*	*R^2 = 0.15*	*pv = 0.01*		*F = N/A*	*df = 39*	*R^2 = 0.00*	*pv = N/A*		*F = 4.37*	*df = 39*	*R^2 = 0.10*	*pv = 0.04*
%△ Vastus Lateralis MT														
Intercept	11.57	1.24	9.34	<0.01	Intercept	11.57	1.24	9.34	<0.01	Intercept	11.57	1.24	9.34	<0.01
	F = N/A	*df = 39*	*R^2 = 0.00*	*pv = N/A*		*F = N/A*	*df = 39*	*R^2 = 0.00*	*pv = N/A*		*F = N/A*	*df = 39*	*R^2 = 0.00*	*pv = N/A*
%△ Rectus Femoris MT														
Intercept	8.36	1.19	7.03	<0.01	Intercept	8.36	1.19	7.03	<0.01	Intercept	14.80	3.04	4.87	<0.01
										IL-6	-0.62	0.27	-2.28	0.03
	F = N/A	*df = 39*	*R^2 = 0.00*	*pv = N/A*		*F = N/A*	*df = 39*	*R^2 = 0.00*	*pv = N/A*		*F = 5.21*	*df = 39*	*R^2 = 0.12*	*pv = 0.03*

Table 12. Backwards elimination regression final output between initial, late and total changes in inflammatory markers in strength and MT.

	Initial % change					Late % change					Total % change			
	Estimate	SEM	t-value	p-value		Estimate	SEM	t-value	p-value		Estimate	SEM	t-value	p-value
%△ 1RM (combined)														
Intercept	32,97	3,30	10.00	<0.01	Intercept	32.97	3.30	10.00	<0.01	Intercept	37.85	3.87	9.78	<0.01
										IL-6	-0.08	0.04	-2.17	0.04
	F = N/A	*df = 39*	*R^2 = 0.00*	*pv = N/A*		*F = N/A*	*df = 39*	*R^2 = 0.00*	*pv = N/A*		*F = 4.37*	*df = 39*	*R^2 = 0.11*	*pv = 0.04*
%△ Vastus Lateralis MT														
Intercept	11.57	1.24	9.34	<0.01	Intercept	11.57	1.24	9.34	<0.01	Intercept	11.57	1.24	9.34	<0.01
	F = N/A	*df = 39*	*R^2 = 0.00*	*pv = N/A*		*F = N/A*	*df = 39*	*R^2 = 0.00*	*pv = N/A*		*F = N/A*	*df = 39*	*R^2 = 0.00*	*pv = N/A*
%△ Rectus Femoris MT														
Intercept	8.36	1.19	7.03	<0.01	Intercept	8.36	1.19	7.03	<0.01	Intercept	10.06	1.40	7.17	<0.01
										IL-6	-0.03	0.01	-2.08	0.04
	F = N/A	*df = 39*	*R^2 = 0.00*	*pv = N/A*		*F = N/A*	*df = 39*	*R^2 = 0.00*	*pv = N/A*		*F = 4.40*	*df = 39*	*R^2 = 0.10*	*pv = 0.04*

Legend: initial % change (week 4 minus baseline values); late % change (week 8 minus week 4 values); total % change (week 8 minus baseline values)

6.4 Discussion

We found no differences between groups regarding IL-6, hsCRP and TNF-α, after 8 weeks of a RE protocol. Furthermore, no inflammatory marker shared a significant amount of variance with changes in strength or MT. These results are in agreement with

our previous results (13), with these leucine metabolites displaying no effect insofar as reducing creatine kinase, a proxy marker of muscle damage. Previous research studies observed that HMB-Ca might attenuate increases in IL-6 (6) while others have failed to confirm those results (8, 9, 21).

The few studies in which C-reactive protein was assessed (not using a high sensitivity assay) have failed to observe any significant outcomes with HMB-FA (8, 11, 12) which is in agreement with our findings. Insofar as TNF-α is concerned, both acute supplementation (few hours post-exercise) and a 23 day intervention protocol, might reduce this biomarker (7, 21), with no longer duration studies so far as we are aware. No research studies regarding inflammatory markers and α-HICA have been performed to date, therefore our results are novel and present a valuable contribution to the current body of evidence.

A recent review has suggested that acute HMB supplementation might attenuate the pro-inflammatory response following an intense bout of RE in athletes (22). In fact, when longer duration studies are conducted (i.e. seven weeks), no effect of HMB was found upon several inflammatory markers (23), which is again in agreement with the data reported herein.

Previous hypotheses regarding the inhibition of ROS (7) with HMB and some research in humans has also observed positive effects upon complement receptor 3 (CR3) expression in monocytes (24) and/or changes in leucocyte binding and adhesion (21). Some studies have reported an attenuation in inflammatory markers, this did not translate into improved recovery or performance (7). Our results do not support an attenuation in any inflammatory marker with either leucine metabolite when comparing to placebo. Furthermore, this did not seem to impair training induced skeletal muscle thickness. Thus, previous hypothesis proposing that increased inflammation might stimulate muscle proteolysis and modulate protein turnover (25), seem unjustified at this point, at least in healthy subjects.

Our results are in direct and sharp contrast with previous research conducted for 12 weeks (9). However, this research study measured cytokines acutely (immediately post exercise and 30 min into recovery) and used a supplement containing other compounds besides HMB-Ca (i.e. glutamine, arginine, etc.) which might have confounded the results,

while cytokines in our study were measured after a 12-hour rest, using a supplement that only contained one of the aforementioned leucine metabolites. These differences might have contributed to some of these discrepancies and strengthen the hypothesis that acute cytokine elevation is likely the result of strenuous exercise-induced tissue damage (26).

Recent research has reported an important action of myokines regarding skeletal muscle adaptation from RE (27). In fact, inhibiting these molecules might impair muscle hypertrophy and strength gains in response to chronic RE in young individuals (28). It seems indisputable that both exercise induced muscle damage (29), enhanced ROS production (30) and inflammation (2) are part of a complex system resulting in skeletal muscle growth. Conversely, chronic excessive inflammation might lead to muscle atrophy, especially under certain disease states (2). There is wide discrepancy in the quantification of these molecules induced by exercise, possibly due to timing of sample collection, sample processing, calculation and other factors (31).

Here we present data regarding leucine metabolites and inflammatory responses to RE. Due to the lack of research pertaining these metabolites and inflammation, we propose that our findings are noteworthy. No leucine metabolite attenuated the elevation of inflammatory markers IL-6, hs-CRP and TNF-α in response to RE. Additionally, changes in circulating inflammatory markers are not related to changes in muscle hypertrophy or strength, in young resistance trained individuals.

Acknowledgments

FJT participated in all laboratory evaluations and wrote the manuscript. CNM, CPM, MJV, JFR, assisted on measurements and development of the manuscript. RWM performed all multiple regression analysis and assisted on the manuscript development. FA, LBS and SMP reviewed and assisted on the manuscript development. All authors approved the final version of the manuscript. All supplements were freely donated by Body Temple, Lda.

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Conflict of interest

At the time of data collection, FJT withheld a position as technical manager for Body Temple, Lda, a company that sells HMB-Ca and HMB-FA.

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CHAPTER 7

Effects of Alpha-hydroxy-isocaproic Acid Upon Body Composition in a Type I Diabetic Patient With Muscle Atrophy – A Case Study[4]

[4]Teixeira FJ, Matias CN, Monteiro CP, Howell, SL, Kones R. Effects of Alpha-hydroxy-isocaproic Acid Upon Body Composition in a Type I Diabetic Patient With Muscle Atrophy. *The Yale journal of biology and medicine. 2018;91(2):161-171*

Effects of Alpha-hydroxy-isocaproic Acid Upon Body Composition in a Type I Diabetic Patient With Muscle Atrophy

Filipe J. Teixeira, Catarina N. Matias, Cristina P. Monteiro, Scott L. Howell and Richard Kones

Abstract

Research involving dietary supplement interventions for sarcopenia and osteopenia in type 1 diabetes patients is scarce. Here we present a case study of a type 1 diabetic patient that was treated with supplemental α-hydroxy-isocaproic acid (α-HICA) for 120 days. Several measures of body composition by dual x-ray absorptiometry, blood markers, and maximum voluntary contraction parameters were assessed at baseline and after 120 days. The patient's baseline weight was 73.2 kg, which increased to 75.2 kg by the 120-day assessment. Salient mass distribution changes included increases of trunk fat mass (+0.4 kg), trunk fat free mass (+2.3 kg), total trunk mass (+2.7 kg), and a decrease of 0.8% in trunk fat mass contribution. Handgrip strength increased by 58.84 N, whereas isometric force in the leg press decreased by 347.15 N. Amelioration of BMD Z-scores from -0.7 to 0.5 and T-scores from -1.0 to -0.9 were noted. Importantly, full hematologic measures and weekly nutritional counselling assessments revealed no signs of adverse effects with α-HICA supplementation. Due to the imperative of maintaining FFM, strength and bone mass in these patients, additional research is necessary to confirm these promising results and to clarify whether leucine and/or one of its derivatives might be clinically useful.

Key Words: *alpha-hica, diabetes, body composition, strength, leucine, intervention*

7.1 Introduction

Diabetes mellitus (DM) is global pandemic of chronic hyperglycemia characterized by diminished insulin secretion and/or low sensitivity in target cells, and an increase in hepatic glucose production (1). Worldwide, the prevalence of DM is currently estimated at 415 million, or 8.3% of the global population (2). The current trajectory predicts this burden will increase to 642 million people with the disease by 2040 (3). Type 1 diabetes represents 5-10% of all DM cases, a figure that is progressively rising so that it too is regarded as an emerging epidemic in several countries (4). This form of the disease is typically expressed during adolescence, but also occurs in older adults (5). One of the severe consequences of DM is myopathy, associated with loss of both skeletal muscle mass and physical capacity (6). Due to the important anticatabolic action of insulin in the ubiquitin proteasome pathway (UPP), both types of diabetes influence skeletal muscle mass (SMM), but differently, with type I resulting in greater SMM loss (7). Interest in DM-associated skeletal muscle mass loss is high for two major reasons: 1) SMM is the major reservoir of post-prandial glucose uptake targeted by insulin and 2) loss of SMM leads to a dramatic reduction in global protein, which might impair mobility and response to critical illness (6, 8).

Leucine metabolites are thought to decrease muscle protein breakdown by inhibiting the UPP (9). α-hydroxy-isocaproic acid (α-HICA) is an end product of leucine metabolism in human tissues (10) with natural occurrence in several foods (11). It has been regarded as an anti-catabolic substance in both in vitro and animal research (12, 13). In vitro research suggests that a possible mechanism for this effect is the inhibition of metalloprotein enzymes (13). However, human α-HICA research is scarce, with only one double-blind human study performed involving 15 young Finnish male soccer players (14). In this work, the researchers reported that administration of 500 mg of α-HICA, 3 times a day, over 4 weeks, led to a significant increase in body weight and whole body lean mass, while fat mass (FM) remained constant. Theoretically, several mechanisms could explain how both leucine or its downstream metabolites might be beneficial to individuals bearing DM (7, 9). Without additional confirmatory research, no definitive statement can be made. Fortunately, the safety of leucine and its derivatives is well established, even in elderly individuals, which permits further research with this

compound in diabetic patients (15). Within this context, we present a case study of a patient with type I diabetes, in which, α-HICA presented favourable changes in fat free skeletal mass.

7.2 Case Presentation

A 59-year old, nonsmoking man diagnosed with type 1 diabetes for 30 years by his primary care physician, presented to our lab. The subject is sedentary with minimal physical activity due to serious mobility limitations which precluded any type of training or exercise on his daily routine. Medical records showed loss of body weight and presumably muscle mass for over one year. The patient had a long history of successive hypoglycemic crises, one associated with cardiac arrest. Fifteen years before, the patient suffered a grand mal seizure that caused permanent damage to the right hip, eventually requiring a complete hip replacement. The primary care physician believed that the combination of impaired ambulation and type 1 diabetes was the primary cause for the patient's more recent insidious muscle loss, especially in the lower limbs.

The patient used slow action insulin (Lantus®-Generis Farmacêutica SA, Amadora, Portugal) ≈25 IU upon awakening depending on glycemic values and fast acting insulin (Humalog®-Lilly Portugal, Lisboa, Portugal) ≈3 IU one hour after meals, also depending on glycemic values. The patient had a flash glucose monitoring system which was alternated between the right and the left arm. Additional medications included pregabalin 100 mg twice a day Lyrica® (Pfizer Ltda, Freiburg, Germany) for peripheral pain and the antiplatelet drug clopidogrel (KRKA, Cuxhaven, Germany) 75 mg before sleep to prevent arterial thrombosis.

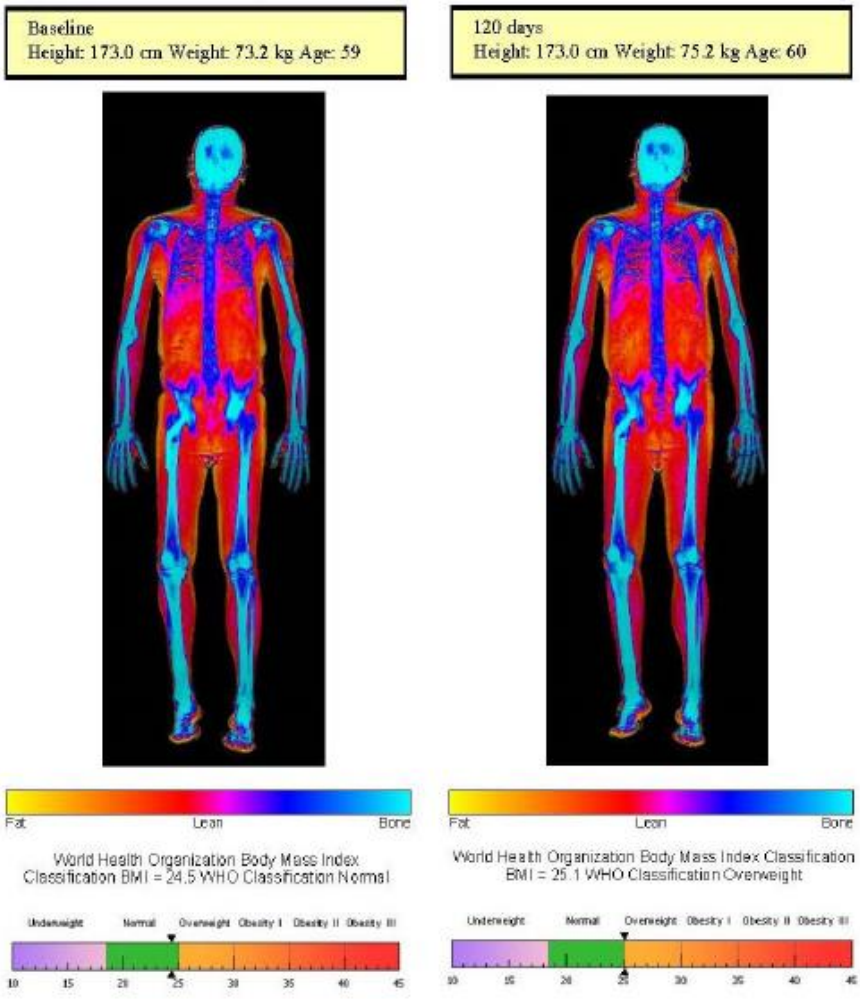

Figure 21. DXA Baseline vs. 120 days

7.3 Measurements

The subject was evaluated at baseline and after 120 days of supplementation with alfa-HICA (from April to August 2017). Evaluations included blood analysis and body composition, and strength measures. All body composition measurements were made after a 12 h fast. A meal replacement bar (Matrix Bar, Olimp Labs, Pustynia, Poland) was provided prior to the strength tests, comprised of 258 kcal (energy) = 26.2 g (protein) + 20.5 g (carbohydrates) + 8 g (fat). During the whole intervention the subject received guidance from his doctor and a registered dietitian. Subject was instructed not to change his physical activity during the intervention, maintaining only his professional daily activity as an administrative assistant.

ANTHROPOMETRY

Height was measured to the nearest 0.1 cm with a stadiometer (Seca, Hamburg, Germany), using standardized procedures (16). Body mass was assessed to the nearest 0.1 kg using a weight scale (Seca, Hamburg, Germany).

Table 13. Body composition characteristics: Baseline vs. 120 days.

DXA	FM-B (g)	FM-120 (g)	AbsC (g)	FFM-B (g)	FFM-120 (g)	AbsC (g)	TM-B (g)	TM-120 (g)	AbsC (g)	Fat-B (%)	Fat-120 (%)	Abs(
L Arm	820	858	+38	3023	3003	-20	3843	3861	+18	21.3	22.2	+0.9
R Arm	691	729	+38	3626	3247	-379	4318	3977	-341	16.0	18.3	+2.3
Trunk	10577	11005	+428	27380	29639	+2259	37957	40644	+2687	27.9	27.1	-0.8
L Leg	2037	2020	-17	8699	8295	-404	10736	10315	-421	19.0	19.6	+0.6
R Leg	1989	2086	+97	8473	8258	-215	10462	10344	-118	19.0	20.2	+1.2
Subtotal	16114	16699	+585	51201	52442	+1241	67316	69140	+1824	23.9	24.2	+0.3
Head	1062	1065	+2	4185	4153	-32	5247	5218	-29	20.2	20.4	+0.2
Total	17176	17764	**+588**	55386	56595	**+1209**	72562	74359	**+1797**	23.7	23.9	**+0.2**

Abbreviations: DXA-Dual X ray absorptiometry, B-Baseline, AbsC-Absolute change, FM-Fat mass, FFM-Lean + BMC, TM-Total mass, % Fat- Percent fat contribution, L-left, R-right

DUAL X RAY ABSORPTIOMETRY (DXA)

The patient underwent a whole-body DXA scan on a Hologic Explorer-W, fan-beam densitometer (Hologic, Waltham, Massachusetts, USA) according to the manufacturer guidelines (17). The DXA scan included whole body measurements of bone mineral content (BMC), bone mineral density (BMD), absolute and relative FM, and fat free mass (FFM). The equipment measures the attenuation of X-rays pulsed between 70 and 140 kV synchronously with the line frequency for each pixel of the scanned image. A step phantom with six fields of acrylic and aluminum of varying thickness and known absorptive properties was scanned to serve as an external standard calibrator for the analysis of different tissue components. The same technician positioned the patient, performed the scan, and executed the analyses (software QDR for Windows version 12.4, Hologic, Waltham, Massachusetts, USA) according to the operator's manual using the standard analysis protocol. The coefficients of variation in our laboratory, based on 10 young active adults (five males and five females), is 1.6% for BMC, 1.7% for FM, and 0.8% for FFM (18).

STRENGTH

Before each assessment the participant was familiarized with the specific strength test. Maximal isometric forearm strength was determined using a hydraulic hand dynamometer model 5030J1(Jamar, Sammons Preston, Inc, Bolingbrook, IL, U.S.A.) with visual feedback (19). The dynamometer was adjusted to the subject's dominant hand with each trial lasting approximately 5 seconds. The best of three maximal trials was recorded to the nearest 2 kg (19.61 N). The same adjustment of the dynamometer was used for all tests. The evaluation of the maximal lower strength was made performing three rapid maximal voluntary isometric contractions (MVC's).

Table 14. BMD T and Z scores: Baseline vs. 120 days.

Score	Baseline	120 days	Critical Values
T-score (% age matched)	-1.0	-0.9	> -1 Normal
Z-score (standard deviations)	-0.7	-0.5	> 2

Table 15. Strength measures: Baseline vs. 120 days.

Test	Baseline	120 days
Handgrip (N)*	480.53	539.37
Leg press (N)*	2954.63	2607.48

*Best out of 3 attempts

The evaluation of the maximal knee extension strength was performed on a custom-made horizontal leg press device (Model 4090E; HBP Exclusive Line) instrumented with an aluminum platform equipped with 4 load cells (Shear Beam Load Cell - Flintec BK2). During the test, the patient was positioned with the hip and knee joints at angles of 100˚ and 110˚, respectively. He was then instructed to produce the maximal force as quickly as possible and sustain the effort for 3 seconds. The force signal was A/D converted (MP100 – Biopac Systems Inc, 16 bits) with a sample rate of 1 KHz. AcqKnowledge software (Biopac Systems Inc) was used to analyze the highest value across the 3 MVC's to the nearest 2.0 N/mV. All strength measures were performed in a fed state (a meal replacement bar was provided to the patient 30 minutes prior to testing).

DIET

The patient followed a diet designed by a registered dietitian from a previous hospital admission. The diet comprised 2200 kcal (138 g protein ≈25% TDEE, 303 g carbohydrates ≈55% TDEE, and 49 g fat ≈ 20% TDEE) and composition was assessed from baseline every 30 days through three-day food records (20) (three non-consecutive days, one being a weekend day) using certified software (Food Processor, Esha Research, Inc, Salem, Oregon, USA). Weekly Nutrition counselling was provided by a registered dietitian to enhance compliance with the hospital diet.

SUPPLEMENTATION PROTOCOL

The supplement consisted of 500 mg α-HICA per tablet (HICA, Onsalesit, SA, Funchal, Portugal). The patient was instructed to take 1 tablet (500 mg) each day at breakfast, lunch, and dinner, with a liquid beverage, as indicated by Mero et al. (14). The daily dosage was provided in individual bags (three tablets per bag). Compliance was assessed when the patient returned empty bags to the dietitian during the weekly nutrition counselling session.

BLOOD ANALYSIS

Blood samples were taken under physician order at a local hospital and processed onsite in a certified laboratory. Venous blood was withdrawn from the antecubital vein after a 12 h fast and analyzed with automated equipment according to standard procedures.

7.4 Results

The patient achieved 98.4% compliance with the supplementation protocol during the 120-day treatment period. At baseline, the patient's weight was 73.2 kg, which increased to 75.2 kg by the 120-day assessment (figure 24). Table 13 depicts regional body composition changes comparing baseline and 120 days. During that same period, the absolute FM, FFM, and total mass increased by 588, 1209, and 1797 g, respectively, along with a 0.2 percent increase in fat. Salient mass distribution changes included

increases in trunk FM of +428 g, trunk FFM of +2259 g, total trunk mass of +2687 g, with a decrease of 0.8 percent in trunk fat contribution.

Absolute change in total bone area decreased by 67.78 cm², whereas absolute change in BMC and BMD increased by 1.38 g and 0.036 g/cm², respectively. Thoracic spine area decreased 34.08 cm², while lumbar spine area increased 19.76 cm². Right leg BMC decreased 24.39 g, while the BMC increased 21.21 g and 18.86 g in the pelvis and lumbar spine, respectively. BMD decreased 0.520 g/cm² and 0.117 g/cm², but increased in all other areas.

Redistribution of BMC in all areas lead to a slight amelioration of BMD Z-scores from -0.7 to -0.5, with T-scores following this trend changing from -1.0 to -0.9.

Table 14 lists the change in T- and Z-scores from baseline to 120 days. Handgrip strength increased by 58.84 N, whereas isometric force in the leg press decreased by 347.15 N (Table 15). Blood markers changes from baseline to 120 days are presented in Table 16. No salient changes in blood markers were noted. Estimated dietary intake from baseline to 120 days is presented below (Table 17). A slight increase in energy was noted (+340 kcal) mainly due to an increase in fat intake (+27 g).

Table 16. Blood composition changes baseline vs. 120 days.

Marker	Baseline	120 days	Change
Hemoglobin (g/dL)	14.6	14.7	0.1 ↑
Erythrocytes x (10^6/µL)	4.83	4.86	0.03 ↑
Hematocrit (%)	44.8	45.0	0.2 ↑
Mean corpuscular volume (fL)	92.8	92.5	0.3 ↓
Red cell distribution width (%)	12.4	12.2	0.2 ↓
Platelet count x (10^3/µL)	298	301	3 ↑
Ferritin (ng/mL)	80.9	79.0	1.9 ↓
Transferrin (mg/dL)	241.0	233.4	7.6 ↓
Serum iron (µg/dL)	78	86	8 ↑
Glucose (mg/dL)	217	252	35 ↑
Glycated hemoglobin (%)	9.6	9.3	0.3 ↓
Median glucose (mg/dL)	229	220	9 ↓
Insulin (µIU/mL)	10.9	11.2	0.3 ↑
Total cholesterol (mg/dL)	193	192	1 ↓
LDL cholesterol (mg/dL)	103	100	3 ↓
HDL cholesterol (mg/dL)	62	64	2 ↑
Triglycerides (mg/dL)	141	139	2 ↓
Albumin (g/dL)	3.7	3.7	=
C reactive protein (mg/dL)	0.182	0.123	0.059 ↓
Uricemia (mg/dL)	6.4	7.0	0.6 ↑
Creatinine (mg/dL)	1.62	1.52	0.1 ↓
Aspartate aminotransferase (U/L)	21	26	5 ↑
Alanine aminotransferase (U/L)	28	31	3 ↑
Gamma-glutamyl transpeptidase (U/L)	37	47	10 ↑
Alkaline phosphatase (U/L)	92	87	5 ↓
Sodium (mmol/L)	142	142	=
Potassium (mmol/L)	5.3	4.9	0.4 ↓
Chlorine (mmol/L)	106.0	105.0	1 ↓
Free testosterone (pg/mL)	12.28	9.39	2.89 ↓

Table 17. Estimated dietary intake from baseline to 120 days.

Table 5. Estimated dietary intake from baseline to 120 days.

	Energy (kcal)	Protein (g)	Carbohydrates (g)	Fat (g)	Protein (g/kg body weight)
Baseline	2200	138	303	49	1.9
120 days	2540	146	318	76	1.9

7.5 Discussion

BODY COMPOSITION

Relevant changes associated with α-HICA administration, in both body weight and FFM, have been previously reported in the literature in 15 healthy young soccer players (14). However, these changes were of small magnitude (+0.3 kg) for both body weight and absolute FFM) in the α-HICA group. The significant increase in FFM was attributed to a variation in the lower extremities (0.4 kg). Our patient increased body weight by 2 kg and total FFM by 1.2 kg, which is almost 4-fold the previous reported values. In our patient, the main contributor to absolute FFM increase was the trunk area (+2.2 kg), whereas in the previous study lower extremities accounted for the significant differences. In our patient, the trunk also contributed to a major increase in FM (+0.4 kg), which was 0.2 percent higher than at baseline. Differences between our patient and the previously reported study (14) can be attributed to the fact that our patient was older and had a diagnosed metabolic disease, but also to the duration of our study (120 days vs 4 weeks).

The incremented FFM in lower extremities in the previous study may be attributed to participation in soccer with primary use of the lower body as a muscular stimulus. Since our subject was sedentary and substantially limited in the lower body from previous hip replacement surgery, it is plausible that the upper body was more stimulated than the lower body, thus leading to greater muscle mass preservation. Although changes in FM were detected, they were not clinically relevant given the duration of the study.

BONE MINERAL DENSITY

Bone mineral density is an expression of BMC/area (g/cm^2) in which values are typically expressed in relative values: T-scores (standard deviations) and Z-scores (%, age matched) (21). In our patient, only trivial changes in BMD were detected per area,

however changes in Z-score and T-score were noted. Our patient had an initial T-score of osteopenia (-1.0) and after 120 days this value was ameliorated to normal BMD (0.9) (21). The Z-score also followed this trend but did not reach the reference value expected for age (>2) (22). Notwithstanding clinical relevance cannot be established from these changes regarding both Z-score and T-score, due to the short duration of the study. Gains in BMD are important for bone strength; however, they need to be sustained over time. The follow-up schedule after initiation of a new therapy is usually superior to six months, typically 1 year (23). Although the interaction between FFM increase and BMC is well supported from both animal (24) and human studies (25), longer trials are required to establish clinical relevance between α-HICA supplementation and bone health. Nevertheless, plausible mechanisms will be further discussed in the mechanisms section.

STRENGTH

Grip-strength is a practical and informative measure of muscle strength in middle-aged and elderly individuals due to the relationship between grip strength and favorable prognosis in health-related events (19, 26). Key health-related prognostic factors are functional limitation, functional decline, daily living disability (DLD), and mortality (19). Muscle weakness is expected in type 1 diabetics due to FFM loss and neuropathy (27). Our patient increased grip strength by 58.84 N in his dominant side (right-handed), which is quite substantial when compared to the baseline value and healthy population of the same age (28). Due to the importance of strength in DLD, comorbidities and mortality we find these results extremely encouraging, certainly warranting further investigation. Our subject's lower body strength, measured through maximal voluntary isometric contractions, declined -347.15 N, which was expected due to mobility limitations and deficient lower body stimulation. Given the circumstances, whether a strength increase would be noted in the absence of mobility limitations could not be established.

BLOOD MARKERS

No salient changes in blood markers were observed after 120 days of α-HICA supplementation, except for a slight decrease in free testosterone and glycated hemoglobin. A drop in free testosterone (>20%) was observed in our patient (albeit within the laboratory reference range), however this did not seem to negatively impact FFM gains. Adult men with type 1 diabetes have a tendency to hypogonadism, however do not

present different total testosterone levels or impaired pituitary-gonadal axis, only slightly non statistically significant lower free testosterone levels, when comparing with healthy control subjects (29). This is further confirmed in larger studies, with type 1 diabetes patients presenting total testosterone, free testosterone, calculated free testosterone and bioavailable testosterone values in the middle of the normal range (29). Since fluctuations within a normal physiological range do not influence muscle protein synthesis (30, 31), we conclude that this decrease has small clinical relevance in which concerns body composition or strength.

Albeit a slight decrease in glycated hemoglobin (-0.3%) was detected with supplementation, it seems unlikely that this might have significantly influenced strength and body composition. In fact some rodent model studies have suggested that leucine and its downstream metabolites might improve insulin sensitivity (29), however studies in humans also suggest the direct supply of amino acids as one of the main pathways involved (32). Conversely, research in younger subjects with type 1 diabetes mellitus has suggested that leucine supplementation might lead to hyperglycemia with higher exogenous insulin administration being required for glycemic control (33). Since glycated hemoglobin is influenced by small dietary changes (particularly in the last 30 days prior to assessment) (34), seasonal variations (35) and other factors (36), we cannot directly attribute body composition or strength outcomes to this blood marker.

Altogether, α-HICA did not adversely influence blood markers. This finding is consistent with recent studies of leucine and leucine-derivatives (α-HICA included) administered safely to elderly populations (15, 37, 38). Administration of α-HICA for 120 days was well tolerated and produced no adverse effects upon our patient. Our findings confirm the marked safety profile noted in the literature (15, 37, 38); hence, α-HICA may be used in this population with confidence.

DIET

A small energy increase was noted from baseline to day 120. According to food records, this increase was mainly due to an increased fat intake (+27 g), while protein and carbohydrate displayed only minor changes. There is no compelling evidence to support fat intake alone regarding these body composition and strength outcomes, however while ingesting sufficient protein, an energy increase might have contributed to some of the

results reported herein. Notwithstanding some research with leucine derivatives, shows improvements in body composition and strength with no significant increase in energy or macronutrients, both in healthy and muscle-wasting conditions (39). Therefore, the relevance of this 340 kcal increase while supplementing with α-HICA, requires further investigation.

PLAUSIBLE MECHANISMS

In "brittle" type 1 diabetes patients with rapidly changing insulin and glucose levels, protein degradation is enhanced, leading to a highly catabolic state reflected by accelerated muscle mass loss (6, 40). Such enhanced protein degradation contrasts with augmented splanchnic protein synthesis (41), likely attributable to increased availability of amino acids from skeletal muscle protein breakdown (7). Both protein synthesis and degradation require high amounts of energy (41), and in the presence of chronically high circulating levels of glucagon and enhanced hepatic gluconeogenesis (42), a negative energy balance is easily attained, followed by weight loss. Insulinopenic animal models show an increase in UPP activity (43), phenotypically resembling type I diabetics. Therefore, it is reasonable to suspect that inhibiting UPP may lead to higher FFM retention. Both leucine and its downstream metabolites have shown the ability to inhibit muscle protein degradation (9). In fact, recent research indicates that one leucine derivative (β-hydroxy-β-methylbutyrate) might slow protein degradation through an alternative pathway to insulin (44). One possible mechanism for the protective effect of α-HICA upon muscle mass is through insulin-like growth factors (IGF-1), since leucine metabolites have displayed the capacity to enhance IGF-1 expression in skeletal muscle (9), although the evidence is equivocal (33). Patients with type 1 diabetes may also experience reduced expression of IGF-1 (45), which attenuates anabolism, since this factor is involved in the regulation of skeletal muscle mass (46). Another possible mechanism for α-HICA-related muscle mass preservation is cortisol modulation, given the frequent elevation of this hormone in type 1 diabetics. Leucine metabolites have displayed promising effects in both reducing (47) and modulating cortisol (48). This catabolic hormone is linked with increased protein degradation in animal models of type 1 diabetes (49).

Another plausible mechanism is the modulation of inflammatory cytokines by both leucine and downstream metabolites. Leucine has displayed, in tumor-bearing rats, the ability to modulate interleukin-6 (IL-6) levels, leading to protein preservation (50), while other leucine derivatives have exerted promising effects in modulating other inflammatory markers (51). Coincidentally, IL-6 levels are upregulated in several type 1 diabetic subjects (49) and are associated with muscle wasting, presumably due to enhanced myostatin expression (52). Rodent models suggest that elevated levels of IL-6 might induce muscle atrophy from both downregulation of growth factor-mediated intracellular signaling (53) and through IL-6 ligands such as signal transducer and activator 3 (STAT3), since phosphorylation of this signal transducer is elevated in the presence of high levels of IL-6 (54). Cachexic conditions observed in diabetes mellitus (DM) are complicated by the fact that chronic elevation of inflammatory markers such as C-reactive protein (CRP) and IL-6 are not only influenced by skeletal muscle (53), but also by the level of physical activity (55). Acute phase response elevations in CRP and IL-6 follow a downward trend after exercise in healthy individuals (56), whereas in DM, CRP and cytokine levels remain largely unchanged (57).

In wasting conditions, the pattern of immunomodulation of inflammatory mediators and regeneration of muscle tissue differs from acute phase responses to chronic responses (58). Subsequently, some researchers have focused solely on immunomodulation to attenuate inflammation in DM (55). Evidence from clinical trials using interleukin antagonists reinforces the need to focus on methods of controlling inflammatory pathways outside of physical activity, such as dietary, nutraceutical or pharmaceutical interventions, as part of an overall immunomodulatory strategy in managing DM (55).

The changes observed in BMD were intriguing and worthy of further attention. A direct mechanism to explain the influence of α-HICA on BMD is presently unknown. Some amino acids have been linked with bone health but not with leucine or its derivatives (59). In fact, research with leucine metabolites shows no effect in bone mass of healthy young individuals (60). Studies using leucine derivatives in populations with type 1 diabetes are insufficient in the literature. One possible mechanism that might explain amelioration of bone health by α-HICA involves IGF-1. With aging and certain disease states, bone remodeling and formation tends to be seriously impaired due to low

production of growth factors like growth hormone (GH) and IGF-1 (25). Since GH and IGF-I are necessary for osteoblast differentiation and function, decreased systemic and local skeletal production of IGF-I as well as increased levels of growth factor binding proteins might down-regulate bone modeling in older individuals which is not adequate to maintain BMD (61). Given that both aging and type1 diabetes reduce IGF-1 expression, it is possible that α-HICA might present favorable effects through the expression of IGF-1. A positive protein balance could also enhance skeleton health (62).

It has been observed that essential amino acids might improve bone matrix collagen protein (63). In this regard, some amino acids like leucine or its downstream metabolites might present actions through their anabolic properties (64). It is estimated that one-third of bone volume is 50% protein, which is involved in the synthesis of type I collagen through post-translational modifications of several amino acids (65). Another factor that might influence bone health and strength is vitamin D. Albeit not being assessed in this case-study, a recent cross-sectional study suggests that in the Portuguese population the median 25(OH)D is 35.9 nmol/L (66). This value is deemed insufficient and inadequate according to the Endocrine Society and the Institute of Medicine, respectively (67). Hence, it is unlikely that a vitamin D increase might have improved BMD. In which concerns strength, improvements in elderly populations have been reported with serum values ranging between 24.7 nmol/L and 74.9 nmol/L (68), therefore it is not clear whether a seasonal increase in vitamin D would have influenced strength results.

Given that, in some studies, leucine exerted a greater capacity to stimulate muscle protein synthesis and elicited a similar capacity to modulate muscle protein breakdown (69), it is unclear whether the effect of α-HICA would be superior to leucine or to any other downstream leucine metabolite. In fact, some research indicates that a leucine rich diet might be beneficial in reducing protein degradation in adolescents with type 1 diabetes (33). Further research should seek to clarify whether supplementing α-HICA or leucine itself produces any benefit in skeletal muscle mass preservation in patients with type 1 diabetes. To further explore this issue, double blind, placebo-controlled randomized trials are necessary, since this study is obviously a limited one case clinical study.

7.6 Limitations

Due to the long duration of this case study (120 days), intermediate measures for body composition, strength and blood markers should have been performed for additional control, perhaps every 30 days. To establish clinical relevance for BMD gains, longer duration studies are mandatory. This study pertains to a single patient clinical case study; therefore, any conclusions should be drawn with extreme caution recognizing its inherent limitations.

7.7 Conclusion

To our knowledge, this is the first reported case in which a type 1 diabetic patient with documented loss in muscle mass has been studied before and after supplementation with a leucine metabolite. Importantly, in this patient a full panel of blood biomarkers and weekly visits for nutritional counselling revealed no signal of an adverse effect. Salient increases in FFM, especially in the trunk area, hand grip strength, and BMD were noted. Due to the imperative of maintaining FFM and strength in these patients, additional research is necessary to clarify whether leucine and/or one of its derivatives might be clinically useful.

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CHAPTER 8

General Discussion

Leucine metabolites, especially HMB, have been surrounded by controversy (1, 2) with this controversy being, at least partially, related with recently reported increases in FFM and reductions of FM (3, 4). Due to the importance of enhancing performance (sporting context) (1) and FFM (sporting and also clinical context) (1, 5), further research is required to investigate these leucine metabolites in both sports and clinical settings.

Systematic reviews and meta-analysis are broadly considered the top of evidence hierarchy, however they are the reflection only of the quality and quantity of the current body of evidence (1). Thus prospective, randomized, controlled scientific trials (RCT) are considered the "gold standard" to investigate potential effects of dietary supplements. According to Maughan et al. (1) the following guidelines are proposed when designing RCT that could be relevant to athletes or used in a sporting context:

1. To perform an a priori power analysis, guaranteeing an adequate sample size to allow the results to have statistical power.
2. To mimic real-life competition, thus adopting a pragmatic approach regarding the trials.
3. To ensure standardized procedures regarding variables that might influence the results. This might conflict, to a certain extent, with the desired pragmatism previously mentioned and reduce real-life application.
4. To use proper supplementation protocols to optimize any effects (dose and timing of intake).
5. To enforce independent verification of the contents to ensure that the supplement complies with label claims.
6. To assess supplement intake by implementing compliance protocols.
7. To apply a performance protocol that is valid and sufficiently reliable to detect small but potentially meaningful differences.
8. To interpret the results in light of the limitations of the study and the change that would be meaningful to real-life sport.

Having considered these guidelines, we performed two intervention trials giving rise to four research papers, the goal of which was to further investigate several aspects of the leucine metabolites, HMB (both Ca and FA forms) and α-HICA. These leucine

derivatives were directly compared, in young healthy resistance trained men, in three pragmatic, randomized, double-blinded, controlled trials (study 1, 2 and 3 – chapters 4, 5 and 6, respectively). In study 1 (chapter 4), HMB, α-HICA and placebo were directly compared pertaining skeletal muscle thickness (a valid proxy marker of muscle mass), several hormones and a proxy marker of muscle damage (CK). Additional analysis regarding body composition (regional and whole-body FFM and FM) was performed in study 2 (chapter 5), while study 3 (chapter 6) assessed several inflammatory markers (IL-6, TNF-α and hsCRP). Study 4 was performed to assess α-HICA's safety (several blood markers under physician order) and also to further elucidate its effect on strength (isometric strength) and body composition (assessed by DXA) in a type 1 diabetic patient.

Our main research findings, limitations and future prospects are considered in the next sections. A brief discussion in the light of the current body of the literature will also be provided regarding each finding.

8.1 Main research findings and discussion

Early works by Nissen et al. observed promising results regarding the effects of HMB on performance (6), however, these data have been recently questioned in some (1, 2), albeit not all reviews of HMB (7). Some of the extraordinary results with respect to changes in body composition reported by some groups are unprecedented in the literature (3, 4, 8), which warrants a critical in-depth analysis. Our investigation is unique, since we directly compared the efficacy of off-the-shelf commercially available forms of these metabolites *versus* a placebo on resistance trained young men, pertaining muscle thickness, performance, body composition, circulating hormones, muscle damage and inflammation. Additionally, we investigated the effects of α-HICA on a diabetic type 1 patient, further providing evidence regarding these metabolites in clinical populations (where there is a paucity of evidence). We propose that our findings are noteworthy and critically add important new information to the current body of the literature regarding these metabolites.

PERFORMANCE

As reported in studies 1, 2 and 3 (chapters 4, 5 and 6), no differences regarding muscle strength were noted, between subjects consuming leucine metabolites or placebo. Only a training effect was found regarding Wingate peak power, CMJ height and power, 1RM back squat and bench press (chapter 4). Our findings of no effect of these leucine metabolites on strength are congruent with some (9-13) albeit not all (3, 4, 8) previous works using these metabolites. One argument that has been used, was that longer duration studies would be required to detect an effect with HMB (14, 15). Albeit our RET protocol was similar (not identical) to previous works (3, 4, 8) and longer than six week in duration, which has been suggested by Wilson et al. (14), as a reason why we may have been unable to reproduce their findings regarding superior results with HMB-Ca or FA versus placebo.

To put our findings in perspective, Wilson et al. (4) reported gains favouring HMB-FA supplementation over and above a placebo of +9 kg and +13.9 kg for 1 RM bench press and squat, respectively. Similar results were presented by Lowery et al. (3) and Kraemer et al. (8) using both HMB-FA and HMB-Ca. Although the magnitude of performance improvements reported in our investigations (study 1, chapter 4) is similar to the strength increments observed in these studies (3, 4, 8), we observed no differences between groups. Recently, Jakubowski et al. (16), using a protocol that exactly reproduced the previously discussed RET protocols (3, 4, 8), found no differences between equivalent amounts of either leucine or HMB. Moreover, the reported gains in strength (16) were also in line with our findings. Accordingly, with the data reported herein 1, 2 and 3 (chapters 4, 5 and 6), it seems unlikely that any performance benefits would be noted by supplementing with either leucine metabolite, in resistance trained men, when consuming sufficient protein and in an estimated positive energy balance.

It should be noted that previous works failed to report absolute values for energy intake and macronutrients (3, 4, 8), which does not allow assessment of whether the participants were in a positive energy balance and consuming sufficient protein. This bears particular interest, since some research as shown that when sufficient energy and protein is consumed, no increments in strength gains are found when supplementing with leucine (17). In summary, our findings are in agreement with some meta-analysis (18, 19) but not with some systematic narrative reviews (20); however, the conclusions of the

former review (20) is strongly driven by three research studies with extraordinary results, which have been questioned (21-23).

Insofar as α-HICA is concerned the evidence is scarce (24). According to our findings, it does not seem plausible that this leucine metabolite (the natural product of the transamination of leucine) might improve performance above sufficient energy and adequate macronutrient intake, at least in healthy resistance trained men. In the clinical case study (study 4, chapter 7), our participant displayed an increase in handgrip strength with no differences in lower body strength. Albeit strength increases are important in elderly and clinical populations, further randomized controlled trials (RCT) should be performed to confirm this increase and its clinical and functional relevance.

As previously discussed in chapter 2, no studies have compared HMB (Ca or FA) to leucine. Also, some studies suggesting positive effects with HMB in elderly populations have been questioned (25). Furthermore, a recent well conducted systematic review suggests that it is highly unlikely that any benefits would be seen with HMB supplementation (26) or leucine in the absence of resistance training. If any effects are seen with leucine or its metabolites, it is likely that those are trivial and likely only seen in bed rest patients or clinical populations (2). Regarding α-HICA, no studies had been performed to date with this leucine metabolite in clinical populations, which warrant further investigation. Additionally, it remains to be elucidated whether α-HICA would present superior results to HMB (either form) or leucine. It has been reported that leucine supplementation might be beneficial in elderly or clinical populations (27), only when insufficient protein or energy are ingested. In our clinical case study the participant ingested the recommended amounts of energy and protein, albeit in lower amounts than seen in healthy subjects in studies 1, 2 and 3. This further strengthens the notion that if any benefits are seen with leucine or any of its metabolites, they are limited to elderly or clinical populations were lower energy and protein intakes are observed. Also, it remains to be elucidated whether α-HICA or other leucine metabolites may outperform leucine regarding functionality, over and above a well-balanced diet, since there is a paucity of studies regarding these compounds. It cannot be inferred, from a single clinical case study trial (with no comparison to a placebo), that α-HICA might outperform leucine or other leucine metabolites. Further well-designed RCT are warranted to clarify these actions in clinical populations.

BODY COMPOSITION

In studies 1 and study 2 (chapters 4 and 5), we observed no differences between groups regarding whole body composition (DXA) or muscle indices (US), albeit small increases in MT and trunk FFM were detected as a result of the training protocol. Our results are again in agreement with some (9-13, 28, 29) but not all previous studies (3, 4, 8, 30-36) in which HMB metabolites have been studied. When considering the entire body of literature pertaining to HMB, it seems that this metabolite might lead to increases of 0.5-1 kg of FFM when comparing to placebo, especially among untrained subjects (15). However, these results are influenced largely by the previously discussed results of three studies that obtained extraordinary results when comparing to placebo (3, 4, 8). In fact, these results presented in trained or recreationally active subjects are virtually unprecedented in the literature with these metabolites (increases of ≈7.4. to 9.3 kg FFM while losing ≈5.4 kg of FM) and exceed those reported regarding testosterone administration (to increase FFM) and orlistat and sibutramine (37-39). Thus, these results seem implausible and have not been reproduced by other laboratories (16). Possible limitations regarding these studies and others in which changes in lean body mass have been ascribed to HMB or its metabolites include the following:

- In the studies from Wilson et al. (4) and Lowery et al. (3) (using the same control group in each study) there was no control for variations in TBW, which have been shown to affect DXA-measured results of lean body mass (40, 41). To prevent hydration issues we undertook standardization procedures pertaining all DXA assessments, also controlling for TBW using BIS throughout the study.
- Some studies have assessed body composition using bioimpedance (8, 34-36). This method has limitations due to hydration issues, with errors being reported versus the gold-standard 4-compartment model of ≈8-10%, when detecting variations over time (42-44). A letter to the editor (23) questioned the standardization procedures of both DXA and US assessments in the Lowery et al. study (3). Inappropriate standardization procedures increase technical errors during DXA assessments, thus being of the utmost importance (45).

- Some studies (3, 4, 8) did not report absolute intakes for energy or macronutrients, which precludes an objective assessment regarding energy and protein sufficiency.
- Wilson et al. (4) presents a standard deviation regarding FFM for the placebo group that is almost three times the reported effect, suggesting significant variability in the outcome. Additionally, the same standard deviations are presented, for all variables studied (strength, body composition and blood markers) throughout their entire research protocol (12 weeks) - intragroup (albeit values were adjusted to the least square mean), which is quite atypical.
- Mechanisms proposed regarding a possible effect of HMB in reducing FM are derived from *in vitro* or animal studies (46, 47), with research in humans not displaying any effects of leucine over 12 weeks in novice trainees (48). Thus, in trained subjects and without a caloric deficit being reported, no plausible biological mechanism can support the magnitude of FM loss reported in these investigations (4, 8, 49). The control group for the studies by Wilson et al. (4) and Lowery et al. (3) and from a third study (50) derive, per the record of their clinical trial, from the same cohort (albeit not disclosed by the authors) (21).
- Additionally, as a minor point, no rationale seems to support any benefits from other compounds added to HMB in both the Lowery et al. (3) and the Kraemer et al (8) studies (22, 51, 52). Thus, it is unlikely that the addition of these compounds might have offered any benefit or amplified any body composition changes.

Regarding α-HICA, we observed no benefits regarding body composition in young healthy resistance trained individuals. The previous research conducted by Mero et al. (24) observed small gains in the lower extremities in soccer players, who were not performing resistance training and thus are not easily comparable to the participants in our studies.

We detected interesting increases in trunk FFM in our clinical case study (study 4, chapter 7). Since our data are from a case study in an elderly individual with type 1 diabetes, in the absence of any exercise training protocol, with lower, although adequate, energy and protein intake, it remains to be elucidated whether similar results would be obtained with leucine or other leucine metabolite. Regarding bone mineral density, we

also detected promising improvements, however longer duration studies are mandatory to confirm their clinical relevance. Importantly, no adverse effects were reported with this leucine metabolite; thus, our work suggests that this leucine metabolite might be useful in this population.

MUSCLE DAMAGE, HORMONES AND INFLAMMATION

We failed to observe any action of any leucine metabolite regarding plasma measures of hormones, markers of muscle damage or inflammatory markers (Studies 1 and 3, chapters 4 and 6). Increases in several hormones were detected regarding the training protocol, but with no differences between groups. These findings are again in agreement with some (3, 10, 16, 33, 34, 36, 53-55) but not all previous studies (8, 35, 56, 57). It is important to note that any changes of the magnitude we observed, were well within the normal physiological range of these hormones and would have had minor, if any, relevance regarding performance or body composition (58-60). In fact, recent research observed that no correlation seems to exists between the concentration of some anabolic hormones, both in circulation and intramuscularly, and the hypertrophic outcomes to resistance training (61). The changes in hormones concentration in study 1, are likely due to the training protocol and are well within the normal physiological range for these hormones with no relevant physiological significance, at least insofar as body composition is concerned.

Our results, finding no effect of either metabolite on training-induced muscle damage are in stark contrast with a recent meta-analysis by Rahimi et al. (62), suggesting that studies involving HMB supplementation over six weeks duration would find positive effects regarding CK and exercise-induced muscle damage. It has been questioned, however, if muscle damage and muscle proteolysis should be suppressed, since this is an essential tool to remove damaged proteins and promote training adaptations (63).

Insofar as inflammation is concerned, most studies that found an effect of leucine metabolites on inflammation, measured inflammatory markers acutely (64, 65) albeit some studies performed chronic assessments (66). Bearing in mind the current body of literature, it seems that HMB might reduce acute inflammation (in the immediate or first few minutes after exercise); however, in longer duration studies (> 7 weeks) when measured at rest and after an overnight fast, as it was the case of our study, no effect is

observed (3, 4, 33), which is in agreement with our results. It is possible, however, that reducing inflammation post-exercise might not be beneficial to maximize hypertrophy gains (67), while reducing chronic inflammation may be important (68). As plasma volume adaptations to long term training are frequent (69), one limitation regarding most studies performed with several leucine metabolites, is related with the absence of plasma volume variation corrections, which might be misleading regarding the interpretation of some reported values (70).

8.2 Limitations and future prospects

It is important that the main limitations regarding our investigation are acknowledged and that future research directions are discussed.

Main limitations:

- In an effort to design a pragmatic research trial we had supplements that looked different (i.e., liquid capsules versus tablets). Since HMB-FA is presented in liquid capsules and HMB-Ca mainly in powdered form, differences existed between both capsules. Additionally, α-HICA was only available in commercialized tablets. Still, we believe this did not compromise the double-blinding assessment, since both capsules and tablets were unmarked and the participants were only aware that these were leucine metabolites. Additionally, we had to change the supplementation timing protocol in α-HICA to guarantee blinding (studies 1, 2 and 3, chapters 4, 5 and 6).
- Although an a priori power analysis was performed (studies 1, 2 and 3, chapters 4, 5 and 6), it should be noted that slightly different methodologies and protocols (i.e. study duration) were used in the studies that we used in deriving our study power.
- In study 4 (chapter 7), due to the long duration of the case study (120 days), intermediate measures of body composition, strength and blood markers should have been performed, perhaps every 30 days. To establish clinical relevance

regarding bone mineral density gains, longer duration studies in this patient group are clearly needed.

Future directions:

- More longer duration studies (> 12 weeks) would be useful at this point to further investigate these leucine metabolites in trained subjects.
- More studies are recommended to investigate these leucine metabolites in elderly and clinical populations. These studies should involve direct comparisons between leucine metabolites and leucine.
- Further research should be performed to investigate intramuscular concentrations of HMB and also possible inflammatory and hormonal responses to this leucine metabolite.
- The possible antioxidant effects of HMB, as claimed by some reviews (71), definitely warrants further investigation.
- Studies using the same dosing protocol (i.e. daily ingested amount) between α-HICA and HMB, are required to provide and unbiased assessment of these compounds. Whether higher doses of α-HICA would be more effective and safe, cannot be established due to the paucity of studies regarding this leucine derivative.

8.3 Conclusions

An extensive review of the literature (chapter 2) was performed to address main research limitations and directions. No studies were reported in the literature directly assessing comparisons of several leucine metabolites, thus four robust research studies were performed to further contribute to the current body of knowledge in this area. These studies are, in our view, well designed, robust pragmatic controlled trials but with limitations that we acknowledge. Study 1 assessed muscle hypertrophy development, strength/muscle power, several hormones and muscle damage markers. Study 2 further assessed salient changes in FFM and FM. Study 3 further contributed by investigating possible effects of these leucine metabolites regarding chronic inflammation. Lastly,

study 4 assessed the effects of α-HICA, in a type 1 diabetic patient, regarding body composition, strength and several safety biomarkers under physician care. Results from these trials, do not allow us to recommend any of these leucine metabolites in young healthy resistance trained individuals, while consuming sufficient protein and energy, to improve strength/power, body composition, hormonal markers, modulate inflammation or muscle damage. Albeit encouraging, the results obtained in the type 1 diabetic patient further require well designed RCT to provide further confirmatory data.

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